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Development of Complementary Deconstructed Viral Vectors to Protect Crops from Environmental Stressors

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ABSTRACT

Inefficiencies in crop production due to drought negatively affect tens of millions of people across the globe every year. Much of this decrease in fruit production is caused by plant stress response pathways. The ultimate goal of this research is to prove that this stress response pathway can be optimized by the introduction of viral genes coding for silencing RNA's. For both regulatory and environmental reasons, it would not be acceptable to release a plant virus with the ability to infect wild type plants in the natural ecosystem. Therefore, the virus has been deconstructed, meaning that a part that is necessary for reproduction and expression has been removed. In this case, the Tomato Mottle Virus (ToMoV) is being used as a model virus. The Replication Initiator Protein, or REP Gene, has been removed. The REP gene has then been inserted into the plant genome via a plant genetic transformation, allowing the plant to complement the deconstructed viral vector.

To avoid gene silencing mechanisms caused by insertion of multiple gene copies, the plants being tested must be homozygous for the REP gene. The original T0 transformation can insert the gene onto multiple places within the plant chromosomes. To ensure a plant is homozygous, seeds must be taken from a hemizygous plant, which has the transgene inserted as a single gene copy. From there, Mendelian genetics for a diploid plant hypothesizes that 25% of the T1 offspring will be homozygous for the REP gene. The research performed in this thesis involves the genetic segregation testing using polymerase chain reaction (PCR) to identify the presence of the REP gene transformed into tomato plants under the control of a drought-inducible promoter. As of the completion of this thesis, several lines of homozygous plants are

in the final stages of verification for testing of the ability to support the proliferation of REP-deficient viral vectors containing plant protective genes.

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I would like to thank Dr. Curtis for his continued support throughout my time as a member of his lab. His breadth of knowledge and willingness to share with me helped me to grow as a researcher and student in ways that I never thought possible. The opportunity to work with Dr. Curtis is an experience that I will never take for granted as it will continue to stay with me throughout my career and life.

I would also like to thank Natalie Thompson for the countless hours she spent teaching me, training me, and helping my research within this lab to succeed. Without Natalie, I would have been unable to complete much of this project, and I am ever grateful for her willingness to sacrifice so much of her time and energy to assist both myself and others in the lab. Jishnu Bhatt and Aisa Sam are two other graduate students who I wish to extend my gratitude towards as they taught and helped me through different projects in the lab.

I also wish to recognize the chemical engineering department and their devotion to undergraduate research, as well as the Chemical Engineering REU program for allowing me to work in the lab throughout the summer of 2021.

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PREFACE

I was given the opportunity to join Dr. Curtis's lab in August of 2020. I spent most of that first fall semester shadowing graduate students and learning about their work. Much of this time was spent shadowing Jishnu Bhatt. Through these shadowing experiences, I gained knowledge in techniques such as aseptic plant tissue culture, media preparation, and plasmid preparation. I also read many papers, expanding my knowledge on genetic transformation, plant genetics, nanoparticle delivery, and molecular biology as a whole.

In the spring of 2021, I was able to continue shadowing other researchers, and spent most of my time learning about Natalie Thompson's work. I was able to continue learning laboratory techniques such as PCR, gel electrophoresis, Edwards extraction, heat shock transformation, digestion, ligation, primer design, seed sterilization, and many other techniques that I used throughout my time in the lab.

I was then given the opportunity to be part of the Penn State Chemical Engineering REU Program in the summer of 2021. This program gave me the resources to work in the lab full time over the summer. The first half of my summer was spent working with the minimal cloning vector pICOz as a basis for creating a viral infection based on Agrobacterium infection. As the Tomato Mottle Virus is bipartite, it has two separately encapsidated viral components (ToMoVA and ToMoVB). My ultimate goal was for successful digestion of both components and subsequent ligation of that partial tandom 1.5-mer repeat 'launch vectors' for both ToMoVA and ToMoVB into pICOz. Following these procedures, a heat shock transformation into *E. coli*

would be performed to see if colonies could be grown. Progress was made on this project, but I ultimately could not obtain ligation products – in part reflecting the difficulty of working with relatively large DNA components, and in particular when these genetic elements involved tandomly repeated DNA.

The second component of my summer was spent using molecular biology techniques toward developing a homozygous REP positive (REP+) line of tomato plants to be prepared for experimentation. Notably, the REP gene has significant functionality with regard to DNA replication and is 'toxic' such that the generation of a stable REP+ plants has never been accomplished. To overcome this, postdoctoral students (Samwel Kariuki and Aliya Fathima Anwar) created an inducible system so that REP would only be expressed under drought and stress conditions. This process of characterizing the transgenic plants they had regenerated, involved both PCR and dPCR for the different transformation lines that included different lengths of the drought inducible Tas14 promoter. The Tas14 promoter drives the dehydrin gene for protection from drought; the transformation constructs included the 'full length' 2kb promoter (Ly44), the ~1kb promoter (Ly45) and the 0.37Kb promoter (Ly46). By utilizing PCR primers that spanned the promoter into the REP{ToMoV} transgene, I could confirm the presence or absence of the Tas14:REP transgene in the regenerated tomato plants. A new methodology of digital PCR that measures the copy number of the Kanamycin resistance selectable marker gene used in transformation was undertaken by Natalie Thompson. Together, these two PCR methods could follow the transgene copy number toward establishing the desired homozygous REP+ plants. The PCR involved performing a simplified DNA extraction method (Edwards Extraction) that was introduced into the lab by undergraduate researcher Briana Talbot during Spring 2021. The extracted DNA was then either frozen or used immediately in the PCR

amplification for the genomically inserted Tas14:REP genes. Following the PCR, a gel was run so that PCR-amplified bands could be visualized. These gels are all available within the Google Drive Personnel Drive folder.

Based on the assumption of the Tas14:REP gene being adjacent to the kanamycin resistance gene, the combined PCR and dPCR information allowed us to make decisions on moving the most promising transgenic plants forward to the next generation of self-pollinated gene segregation. If a plant was hemizygous, its seeds were planted with the hope of obtaining homozygous offspring. If a T0 initial transformed plant had a copy number of 1, this would correspond to 2 separate gene insertions, where subsequent seeds were planted in the hopes of obtaining a subsequent segregated single-gene copy insertion hemizygous plant that could then be isolated as a homozygous single gene insertion with subsequent segregation generations.

Throughout my senior year, I continued working towards creating this line of homozygous, REP positive plants. Work continued throughout the fall semester. I had to slow down a bit in the spring for thesis and career work but am looking to continue this project until graduation. Ultimately, this overall effort has generated a breadth of different transgenic insertion events and thousands of associated characterized seeds that contain the viral REP functionality under the control of a stress-inducible promoter. These transgenic plants can then be used as a host for the deconstructed REP- viruses as a basis of testing the originally proposed plant protection strategy.

Chapter 1

Introduction

One of the major environmental stressors that is dealt with across the globe in drought. According to the World Health Organization, drought can occur anywhere in the world and is caused by low precipitation totals leading to a lack of water.[1] Drought affects tens of millions of people every year, and threatens hundreds of millions more.[2] The effects of drought can be devastating. In most cases, drought makes it substantially more difficult for certain crops to survive and grow, especially those that have been grown for generations in conditions that provide ample water. This lack of crop growth can lead to malnutrition, usually in the form of micronutrient deficiency. This makes people more susceptible to a range of diseases like cholera and pneumonia and has even been seen to increase mental health disorders. In fact, more than a third of childhood deaths across the world, most of which occur in drought-stricken areas of Africa and South Asia,[3] stem from malnutrition. In extreme cases, drought can force mass migrations of populations who must move in order to survive, resulting in potential political and social turmoil.[1]

Along with hunger and malnutrition issues, lack of agricultural resources can lead to conflict. In fact, the current issue between Russia and Ukraine can largely be attributed to the food crisis. Ukraine is known as the breadbasket of Europe, and has extremely fertile soil that produces about two-thirds of Europe's crops. As Russia seeks to feed their population, and Europe seeks to keep the resources they currently have, the war could have major implications for the global food supply.

As the global climate heats up, these problems are projected to worsen. Climate change will lead to more drastic weather patterns, one of which is drought. As drought increases, technologies and practices must be developed that will allow humans to survive these droughts in sustainable ways, producing the crops they need to feed themselves and the growing human population.

Over the past few decades, scientists have begun responding to certain agricultural needs by taking genetic material from one organism and introducing it into another. This process, known as genetic engineering, has many known benefits to both the farmer and the consumer. Genetically modified organisms have produced higher crop yields at lower costs, all while providing higher nutrient levels than their organic counterparts. The DNA of some crops have also been altered to provide benefits such as insect and disease resistance, reducing the need for pesticides, making them more environmentally and cost friendly.[4]

That being said, the implementation of DNA modifying technologies has not come without risks. For one, it is relatively common for horizontal gene transfer to occur, introducing traits such as antibiotic resistance to organisms that were not originally intended for this purpose. Vertical gene transfer has also become an issue. For instance, when a certain salmon population was genetically modified, the transgenic salmon were more likely to reproduce, but their offspring were less viable, which threaten the health of the entire population.[4] Because of risks like these, it is of utmost importance that the ability to change the genome of organisms is not taken lightly, but handled with care to protect not only that organism but the well-being of the environment and society around it

Utilizing Viral Vectors to Protect Plants

One emerging technology in the field of genetic engineering is the creation or modification of viruses to make them beneficial to infected organisms. A major benefit to this technique is that the viruses can be designed in such a way that they lack certain components necessary to their vitality outside of a lab environment.[5] This project therefore, focuses on using the emerging field of viral modification and delivery to assist plant response to environmental stressors using drought as a model stressor and the tomato plant as a model species. An example of a tomato plant used in the laboratory can be seen in Figure 1.

Figure 1. Example of a tomato plant used in the laboratory.

When faced with a stressor, plants tend to react much more drastically than is necessary, causing them to reduce productivity far more than is necessary to survive the drought. One major reason for this is that they are incapable of predicting or knowing how long the drought

might last. In the case of tomatoes, a lack of water causes the plant to release hormones, which initiate a signal transduction pathway and ultimately tell the plant to conserve and reallocate resources in a way that is the most evolutionarily beneficial, whether that means increasing their chance at survival or their offspring's chances of survival. The first step in the process is to stop producing fruit, and only use resources to grow a larger root system, hoping to survive until watered again. If the drought endures, and the plant recognizes that death is inevitable, it then transitions to utilizing all of its resources to produce fruit, in a self-sacrificial manner. This increases seed production, but ultimately causes the death of the plant.

If this process can be stopped before the plant response becomes too drastic, the plant can survive for a longer time under normal productivity standards and can continue producing the fruit and staying healthy until water is again plentiful. One way to stop this process is to introduce genes coding for RNases. RNases, which have many gene silencing functions, can be coded for on a virus and delivered to a plant. With these genes now inside the plant cell, the RNases will proceed to shut down the signal transduction pathway, halting the release of drought response hormones, allowing the plant to continue growing and producing as in times of plentiful water supply. Once water is again restored to the plant, it will still be alive and healthy and will continue on as if no stressor had occurred.

The benefit to using viruses as opposed to directly modifying the plant genome for the desired trait is that single gene transformations take months, followed by years of growth, planting, and seed distribution, making it much harder for scientists to respond to environmental stressors in a realistic time frame. Furthermore, use of the most prevalent gene editing technology, Agrobacterium tumefaciens, can only be done in labs under controlled conditions, making plants already in the field vulnerable to environmental changes. On the other hand,

viruses can be introduced by other methods, such as whiteflies. If whiteflies containing certain viruses are released and allowed to feed on plants, they can transfer these viruses to the plants, helping the plants to respond in a much more time efficient manner.

As many viruses are parasitic to their hosts, it is of utmost importance that proper care is taken to ensure that viable viruses do not escape into the environment. To remedy this issue, this project is using only deconstructed viral vectors. This means that certain parts of the virus necessary for function and replication have been removed in order that the virus cannot be replicated under normal conditions. For instance, one virus that is being introduced into plants is the Tomato Mottle Virus, abbreviated as ToMoV. ToMoV is in a class of viruses known as Begomoviruses. Begomoviruses are in a family known as Geminiviridae.[6] One unique aspect of Geminiviruses is that they only have one gene known to be necessary for replication, the Replication Initiator Protein, or REP gene.[7] Therefore, if the REP gene is removed from the virus, it can no longer be replicated on its own.

Once this deconstructed REP negative begomovirus is obtained, it is no longer viable to be used in naturally occurring crops and plants. Transgenic plants therefore need to be created to complement its function. These plants are the ones that must be transformed using Agrobacterium tumefaciens, and this process takes a few years from start to finish. Once in the plant genome however, the replication initiator protein can be used to complement any number of deconstructed viral vectors, depending on the end goal of the addition. If the plant is experiencing drought, a virus that helps increase drought resistance can be introduced, whereas if the plant is experiencing a biotic pressure, a virus containing resistance to that biotic stressor could be introduced. This process is much more time efficient than trying to change the genome

of the entire plant, ultimately a difference of months and years, allowing plants that are already in the field to be protected from different conditions and circumstances that they may face.

One issue with this technique is that, as the replication initiator protein is not naturally occurring in the plant, it can be toxic. To alleviate this problem, the REP will be downstream of a stress inducible promoter, rather than a constituent promoter, and therefore only translated when the plant is under some sort of stress. For example, in the case of ToMoV, the REP will be taken out of the virus but instead be present in the plant attached to the Tas14 promoter, a promoter that is induced by the presence of abscisic acid that is released during drought or other stresses. If ToMoV is introduced during times of normal environmental conditions, it will not be expressed, as its replication initiator protein is not expressed in the plant. On the other hand, in times of drought, the Tas14 promoter will be turned on, causing the ToMoV REP in the plant to be expressed and ultimately allowing the deconstructed Tomato Mottle Virus to be expressed. Figure 2 shows a schematic of the REP gene on the Tas14 promoter.

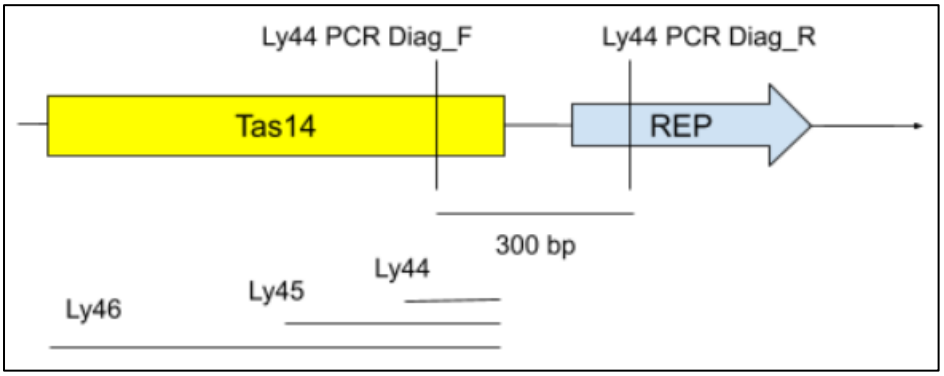


Figure 2. The Replication Initiator Protein (REP) gene on the Tas14 promoter.

The Ly44 plants were transformed using a 2000 base pair promoter, the Ly45 plants were transformed using a 1000 base pair promoter, and the Ly46 plants were transformed using a 369 base pair promoter. PCR was designed to amplify the 300 base pair region between the two primers, Ly44 PCR Diag_F, and Ly44 PCR Diag_R.

In order to transport the virus in the field, whiteflies will be used as a vector. To inoculate the whiteflies, they will feed on plants that have the deconstructed virus, negative for both REP (REP-) and the Coat Protein (CP-), which allows for virus encapsidation and movement. The plants that have the viruses will have both the REP and CP, in order to allow expression of the virus and subsequent uptake by the whiteflies. Once the whiteflies have the deconstructed virus, they will go and feed on plants that are REP+ but CP-, in order that the virus can be expressed in these plants and move throughout the plants, but cannot be encapsidated and moved to other whiteflies or other plants. This will allow for beneficial viral traits to be expressed in plants all while stopping any unintended movement or targets of the virus.

The ultimate goal of this project is a proof of principle designed to deliver a beneficial trait and allow plants to survive longer and more productively under certain stressors. Tomato has been chosen as a model plant, as it is a $96 billion industry in the United States alone, all while being susceptible to over 160 viruses. On top of this, many of the techniques being studied can be used on other plants, and this deconstructed viral vector complementation is an emerging field that could have lasting ramifications on molecular biology, the agriculture industry, and food supplies across the globe.

Chapter 2

Background and Literature Review

Transforming Plants Using Agrobacterium

Over the past few decades, the most common technique to transform plants was through the use of Agrobacterium. Many species of Agrobacterium have been found to introduce diseases into plants. For instance, *A. rhizogenes* causes hairy root disease, A. rubi causes cane gall disease, and A. tumefaciens causes crown gall disease. This is due to their ability to introduce nucleic acids into the plant cell nucleus, thereby infecting the plant with foreign DNA. Scientists have capitalized on this ability, realizing that if the genetic makeup of Agrobacterium can be altered, these genes can subsequently be added to and expressed by cells of other organisms, such as plants, fungi, yeasts, and even humans.

From a molecular standpoint, Agrobacterium can transfer regions of a tumor-inducing plasmid into the plant's nucleus, causing a transformation of the plant genome. The DNA that is transferred, known as the T-DNA, is processed and exported from the Agrobacterium cell and introduced into the plant cell in large part due to the activity of certain virulence genes. These virulence genes, VirD1 and VirD2 recognize specific border sequences of the transfer region of DNA, and process this region via endonuclease activity. The stand is nicked, resulting in a single stranded region of T-DNA, with polar borders, that will ultimately be introduced to the plant cell.[8]

In order to make this transition to the plant cell, a VirA protein must first sense the presence of phenolic compounds that the plant releases upon wounding. VirA then activates the VirG protein by autophosphorylating and transphosphorylating it, which in turn increases the

transcription levels of the virC1 and virC2 genes. The VirB and VirD4 proteins then create a type IV secretion system in order to facilitate the transfer of the T-DNA by forming the membrane channel and serving as ATPases to fuel the process. The VirD2 protein, which has a nuclear localization signal, binds to the single stranded T-DNA and delivers it to the plant cell nucleus.[8]

One issue faced in cloning desired genes into Agrobacterium in order to transfer them into plants is that the Ti plasmids are very large so there are no unique restriction endonuclease sites where the T-DNA can be inserted. One method to avoid this problem is cloning the gene of interest into a separate plasmid that does have the exact restriction sites, as well as inserting an antibiotic resistance gene into this region. This plasmid can then be introduced into Agrobacterium and selected for by that antibiotic. From here, the transfer region and virulence DNA can be separated onto different replicons so that they can function together to deliver the genes in the Agrobacterium cell to the plant nucleus.[8]

Tomato Mottle Virus

The Tomato Mottle Virus, abbreviated as ToMoV, is a geminivirus infecting tomatoes that was discovered in Florida in 1989. This virus, known to stunt plant growth and cause mottling and upward curling of the leaves, was estimated to reduce the tomato crop value by $125 million in that year alone, in some places infecting upwards of 95% of the crop. ToMoV has a similar host range of most tomato geminiviruses, and has been seen to infect tomatoes from the genera Solanaceae and Fabaceae. Whiteflies such as *B. tabaci* were found to transmit ToMoV between tomato plants, but transmission was not then passed on to future generations

through the seeds. Upon experimentation, ToMoV was also able to infect Nicotiana species, as well as the common bean.[9]

The Tomato Mottle Virus is considered a bipartite virus, meaning that it has two segments of DNA used in order for the virus to perform its function. The ToMoVA plasmid contains four genes. The AL1 gene, also known as the REP gene, codes for the replication initiator protein and is necessary for viral replication and function.[10] This REP gene is the only gene that is always necessary for replication of geminiviruses.[7,11] The AL2 gene codes for a transcriptional activator protein and helps suppress RNA-mediated gene silencing.[12] The AL3 gene helps increase viral infectivity by coding for a replication enhancer protein.[13] Finally, the AR1 gene codes for the coat protein, or CP, which encapsidates the virus and is necessary to move it into and out of the nucleus. The LIR is the location of the origin, so replication begins in this region.[14]

As is common in most bipartite begomoviruses, the B component, or ToMoVB in this case, is necessary for the movement of the virus. This contains the BR1 and BL1 genes that are required to spread the virus throughout the plant.[15] A diagram of both ToMoVA and ToMoVB can be seen in Figure 3. Both of these parts of the virus are on the same promoter.

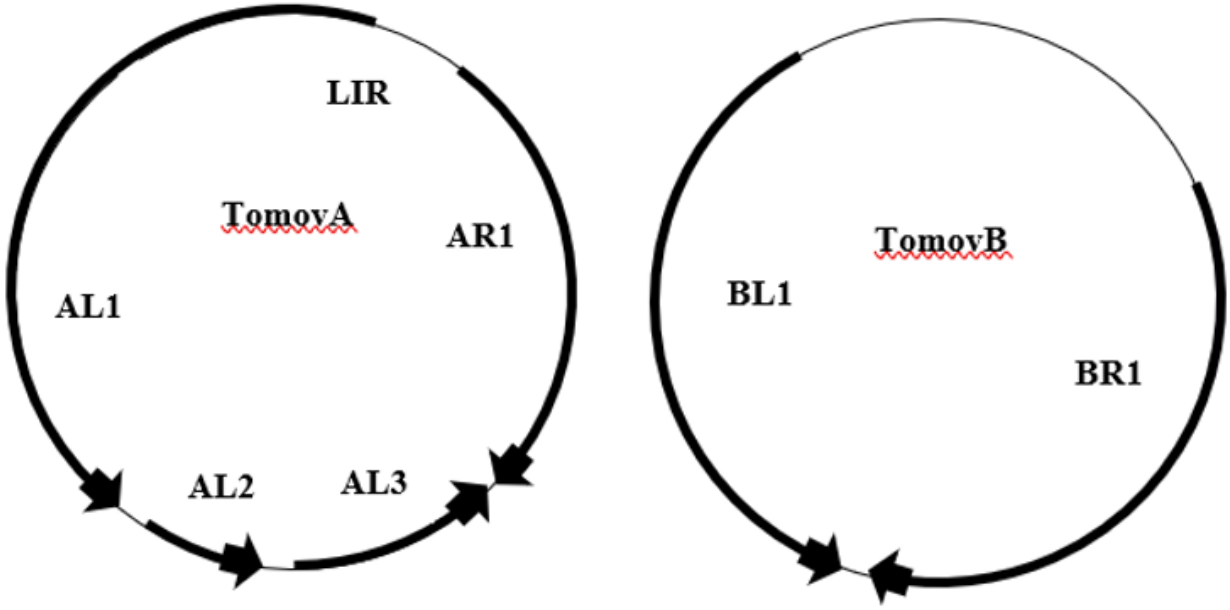


Figure 3. The circular genomes of the A and B components of the Tomato Mottle Virus. AL1 is also known as the REP gene.[16]

Gene Silencing

In order to respond to different stresses, protect themselves from foreign materials, or just generally to grow and function, plants have functions known as gene silencing. This is the process by which plants regulate which genes are expressed at any given time. Gene silencing can occur both at the transcriptional and translational levels depending on which pathways are used.

Generally speaking, this process is performed by microRNAs, or miRNAs, which have been seen to induce mRNA degradation in animal species and induce translational repression in plant species.[17] dsRNA and siRNAs can also contribute to this process, and are both processed

in part by RNases.[18] For plants, this is a crucial way to respond to environmental stresses. For instance, if the plant senses a foreign virus, it can silence the virus and not express the proteins it is coding for. In turn, viruses have developed systems of antigene silencing whereby they find ways around this gene silencing and still get expressed. This is one of the major reasons why Nicotiana benthamiana is so easily transformed by viral DNA, as it does not have strong gene silencing measures compared to other species.

Gene silencing can be a problem in genetic engineering, as plants are likely to silence foreign genes, especially genes that are more abundant. In fact, a correlation seen between higher transgene copy numbers and lower transgene expression has been observed again and again. In some cases, transgene expression was seen in plants hemizygous for the gene but was silenced in plants homozygous for the gene.[19]

Chapter 3

Experiments and Hypotheses

As stated earlier, the ultimate goal of this project is to protect crops from environmental stressors by transforming them to complement deconstructed viral vectors that can be transmitted by whiteflies. To do this, a viral vector must be taken and modified so that it becomes beneficial to the plant. This process will involve removing the harmful aspects of the virus that hurt the plant and replacing them with helpful aspects. These beneficial genes will translate to ATPases, which help process miRNAs. The miRNAs will then interrupt and silence the drought response pathways in the plant, initiated when the Tas14 promoter turns on in response to stress, whereby the plant stops producing fruit for a short time to conserve resources then ultimately produces as much as possible, wasting all of its resources with the expectation of death. In silencing this pathway, the plant continues growing and producing fruit as if it had a normal amount of water, until it can soon be watered again. That being said, this project focuses more on proof of concept, so in many cases the viruses that are considered harmful to the plant will be used.

In working with viruses, it is of utmost importance to protect non-target plants from becoming infected with a live virus. To do this, a deconstructed virus will be utilized, meaning that it cannot be replicated on its own. In this case, the Tomato Mottle Virus will be the main virus under study. To begin, the Replication Initiator Protein, necessary for replication, and the Coat Protein, necessary for encapsidation and transfer, will be removed from the virus. These traits will then be engineered into a small variety of tomato, Florida Lanii, so that the tomato now has the genes for the replication and encapsidation of ToMoV. However, these genes can be toxic to the plant, as they are foreign, so they will be attached to the drought inducible

promoter Tas14, rather than a constitutive promoter, so that they are only expressed during times of stress where ToMoV will be introduced. Therefore, when ToMoV is introduced, and the Tas14 promoter is turned on, turning on the REP and CP genes as well, the ToMoV virus will be replicated and expressed by the plant.

One issue facing the engineering of transgenic plants for the REP and CP genes is that they are foreign and likely to be silenced by the plant. Because of this, it is crucial that the plant contains the genes, but has as low a copy number as possible. The ultimate goal is a copy number of 1. When originally transformed, the plant could take on the gene any number of times, usually somewhere from 0 to 3, at half step intervals. Upon this transformation, the gene could appear once on one chromosome, giving it a 0.5 copy number relative to the Systemin reference gene, once on each chromosome, giving it a 1 copy number, twice on one chromosome, also resulting in a 1 copy number, or any number of times and combinations on each. At the moment, the goal is a homozygous plant, with the gene appearing once on each chromosome. Based on Mendelian genetics, every offspring of this plant would be homozygous as well, making the plant line easy to grow and experiment on. To obtain this copy number of 1, specifically with one gene on each chromosome, hemizygous plants with 1 gene on one chromosome, meaning a copy number of 0.5, must be created and bread with themselves, producing offspring that are 25% homozygous for the trait with a copy number of 1, 50% hemizygous for the trait with a copy number of 0.5, and 25% that don't have the trait at all, with a copy number of 0. The homozygous plants will then be used for experimentation and producing future generations. If the gene is too prevalent in these and is silenced, the same experiments will be attempted on plants with a 0.5 copy number, meaning that each one must be

tested first, involving a lot more work than if the viral complementation is successful in homozygous plants.

Chapter 4

Materials and Methods

Edwards Extraction

The Edwards Extraction method, a DNA extraction method published by K Edwards in 1991,[20] was used to extract DNA from plant tissue to prepare it for PCR. This method first involved making a buffer containing 200 mM Tris HCl with a pH of 7.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS. Plant tissue was taken in a 1.5 mL centrifuge tube and a micro-pestle was used to grind the tissue for 15 seconds. 400 uL of buffer was then added, and the solution was shaken for 5 seconds. The solution then sat at room temperature for an hour. At the end of the hour, the tube was centrifuged for 1 minute at 13,000 rpm, and the supernatant was transferred to a new tube where 300 uL of ice-cold isopropanol was added. After sitting at room temperature for 2 minutes, the solution was centrifuged for 5 minutes at 13,000 rpm. The isopropanol was evaporated off at room temperature, and the pellet was resuspended in 100 uL of TE buffer. This was the final solution that was used in the PCR.

PCR for the Tas14-REP Genes

Plants had originally been transformed by previous lab members including Samwel Kariuki and Aliya Fathima. This added the REP Gene on the Tas14 promoter. Once transformed, these plants were tested for the REP Gene and the Tas14 promoter using PCR. The PCR solution included 10 uL GtAQ Master Mix, 0.4 uL forward primer, 0.4 uL reverse primer, and 9.2 uL of nuclease free water. Originally, primers were used that covered over 1000 base pairs. PCR was continually unsuccessful due to the large amplicon region, so new primers had to be designed. Primers were therefore designed to amplify a 300 base pair region of the genome

spanning the Tas14:REP junction. The sequence of the forward primer, Ly44 PCR Diag_F was GTGCCTCTAATCAGCCTATCC and the sequence of the reverse primer, Ly44 PCR Diag_R was TGGGAAAGTGCTTCTTCTTTAGAC. In this case, the testing was performed on (Ly44) plants that had about a 2000 base pair Tas14 promoter, (Ly45) about a 1000 base pair Tas14 promoter, and about a (Ly43) 369 base pair Tas14 promoter. The primer was therefore designed to attach to all 3 of these promoters, so it could be used to test each type of transformed plant, as the final 369 base pairs of each promoter were the same. Both negative and positive controls were used for each PCR. The negative control was the PCR solution without any extracted DNA. The positive control was a sample of the plasmid containing the Tas14:REP junction directly. This solution had originally been used to transform the plants. In the future, the SYS gene will also be used as a positive control, as this gene should be present in all tomato plants. The PCR cycles were set as seen in Table 1.

Table 1. PCR cycles for amplifying the Tas14-REP junction.

Steps 2-4 are repeated 30 times.

Step	Temperature (°C)	Time (s)	Stage	Reasoning
1	95	180		
2	95	30	Denaturation	Temperature based on GtAQ
3	62.8	30	Annealing	Temperature based on primers
4	72	25	Replication	Time based on amplicon length (1 min/1000bp)
5	72	180		

Gel Electrophoresis

Once a PCR product was obtained, a gel was performed to determine whether or not a band had been amplified. The (1%) gels in this paper were made using 1 g of agarose and 100 mL of TAE buffer, along with 7 uL of gel red as a 300 base pair region was being amplified. However, gels can be made using different concentrations of agarose depending on the size of the amplicon. As a reference, a ladder solution was used in each gel to determine the length of the amplified region.

dPCR

Digital Drop PCR, or dPCR, was also performed by Natalie Thompson to test the copy number of the inserted gene. In this case, dPCR was performed for the NPTII gene rather than the REP gene because this allowed for the use of the expensive dPCR conjugated primers for any transformation event using kanamycin selection. dPCR allowed the gene copy number to be measured by ratio comparison of the DNA level relative to a known tomato plant gene. This procedure has not yet been published within the lab but can be found in the REP Complementation Manuscript Draft in Dr. Curtis's lab files.

Note that in the development of the PCR methods, it was observed that the lack of a PCR product could represent a failure of the DNA extraction, or problematic implementation of the PCR procedure. For this reason, a refined PCR screening methodology was developed that would utilize the PCR primers for the systemin gene (SYS) to allow direct confirmation of the presence of this DNA as positive control for the DNA extraction procedure.

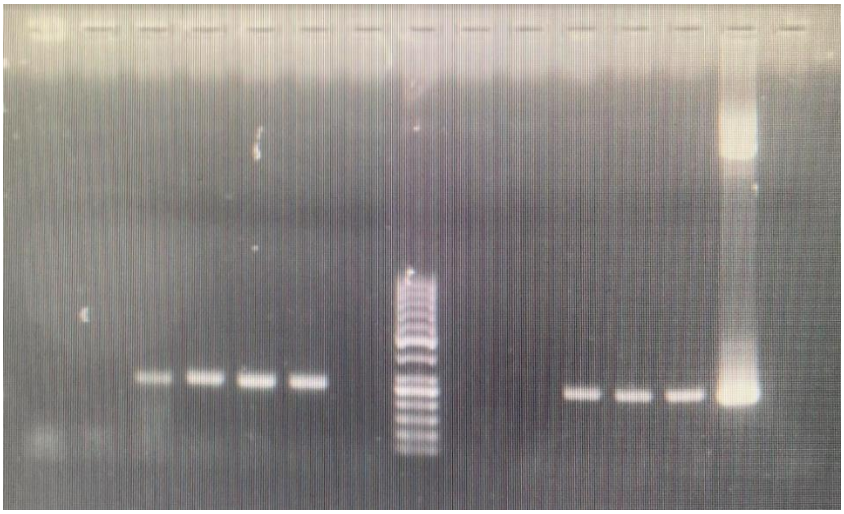
Chapter 5

Results and Discussion

The transgenic plant inventory names were based on the construct designation Ly10 – Ly120 and the generation of the plant. T0 is the initial transformation (e.g. Ly44.1), and its seeds give rise to the next T1 generation (e.g. Ly44.1.3). Ly represents the tomato species under study, *Lycopersicum esculentum*. As noted in methods, the plants originally inserted with the 2000 base pair Tas14 promoter are designated Ly44 and the plants originally inserted with the 1000 base pair Tas14 promoter are designated Ly45. The original transformants, or T0 generation have a decimal and another number. For instance, Ly44.3. Subsequent generations each contain one more decimal point, but keep the same parent format otherwise. For instance, a first generation offspring of the Ly44.3 plant might be named Ly44.3.5. Along with planting seeds and obtaining offspring, sucker clones were also taken from plants. Sucker clones have the exact same genetic makeup as the plant they were cloned from. The naming system of sucker clones involves adding a lowercase b to the name of the plant they were taken from, and adding a number depending on which number clone it was. For example, if a sucker clone was taken from the plant Ly44.3.5, it might be known as Ly44.3.5b1.

To determine whether the Tas14 promoter and Replication Initiator Protein (REP) had been inserted into each plant, an Edwards Extraction and subsequent Polymerase Chain Reaction (PCR) were performed. Gel electrophoresis was then used to test the PCR products for the presence. A band indicated the presence of the Tas14:REP junction and a blank lane indicated the absence of this gene. A positive control, based on the Tas14:REP construct, as well as a negative control are also included in most of the gels. Depending on stocks, the ladder used

differed between gels, but every band seen on these gels occurs at the 300 base pair marking, consistent with the 300 base pair region of interest that was amplified in the PCR step. An example of a gel is seen in Figure 4.



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Lane 1: Ly44.9.5.9
Lane 2: Ly44.9.5.10
Lane 3: Ly44.9.5.11
Lane 4: Ly44.9.5.12
Lane 5: Ly44.9.5.13
Lane 6: Ly44.9.5.14
Lane 7: Ly44.9.5.15
Lane 8: 50 kb Ladder
Lane 9: Ly44.9.5.16
Lane 10: Ly44.9.5.17
Lane 11: Ly44.9.5.18
Lane 12: Ly44.9.5.19
Lane 13: Ly44.9.5.20
Lane 14: Positive Control
Lane 15: Negative Control

Figure 4. Gel for PCR screen of a T2 segregation of the (2Kb)Tas14:REP transgene.

Analyzing this figure, it can be determined that 7 of these plants are positive for the Tas14:REP junction. The same procedures were carried out for many other plant constructs. These gels can be seen in Appendix A. A summary of the gel results can be seen in Table 5.

Using Figure 2, along with data from other gels, the offspring of the hemizygous plant Ly44.9.5, that have Tas14-REP PCR and NPTII dPCR data were compared in Figures 18 and 19 to show whether the Mendelian predictions for diploid segregation hold true for this construct.

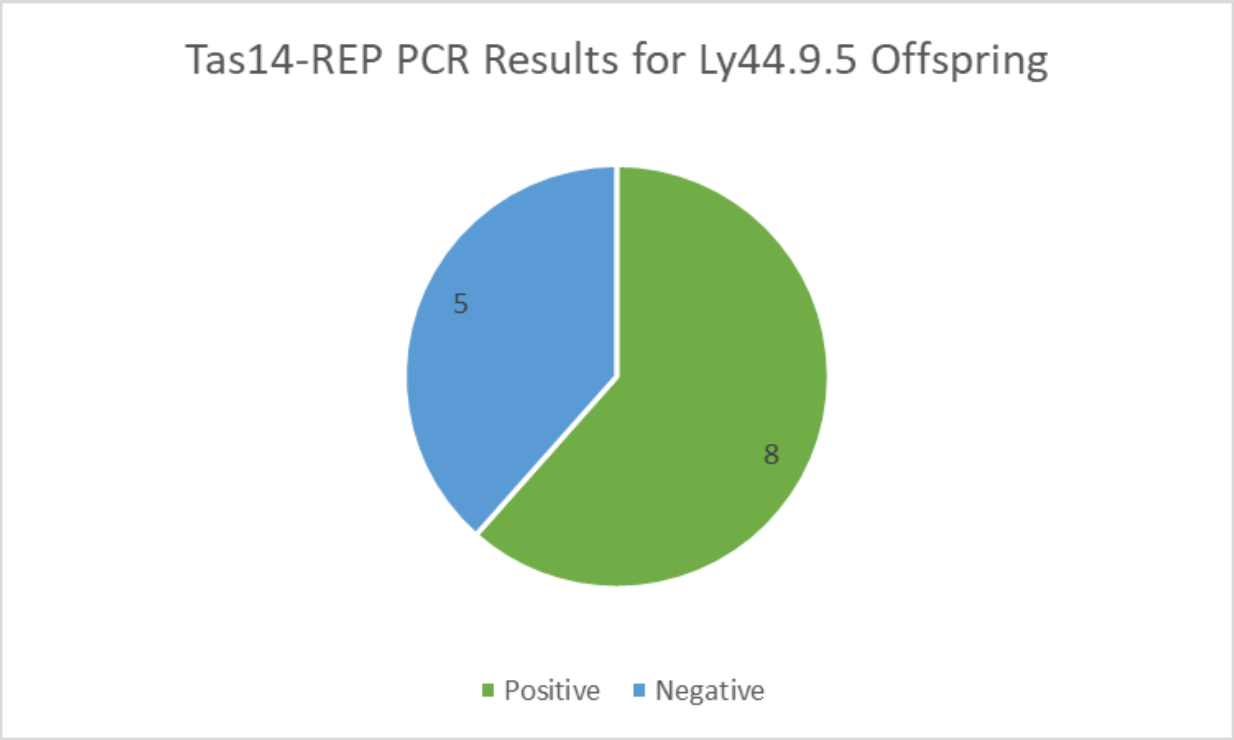


Figure 5. Tas14-REP PCR results for Ly44.9.5 offspring.

Based on Mendelian Genetics, it was predicted that 75% of the plants tested would be positive and 25% of the plants tested would be negative.

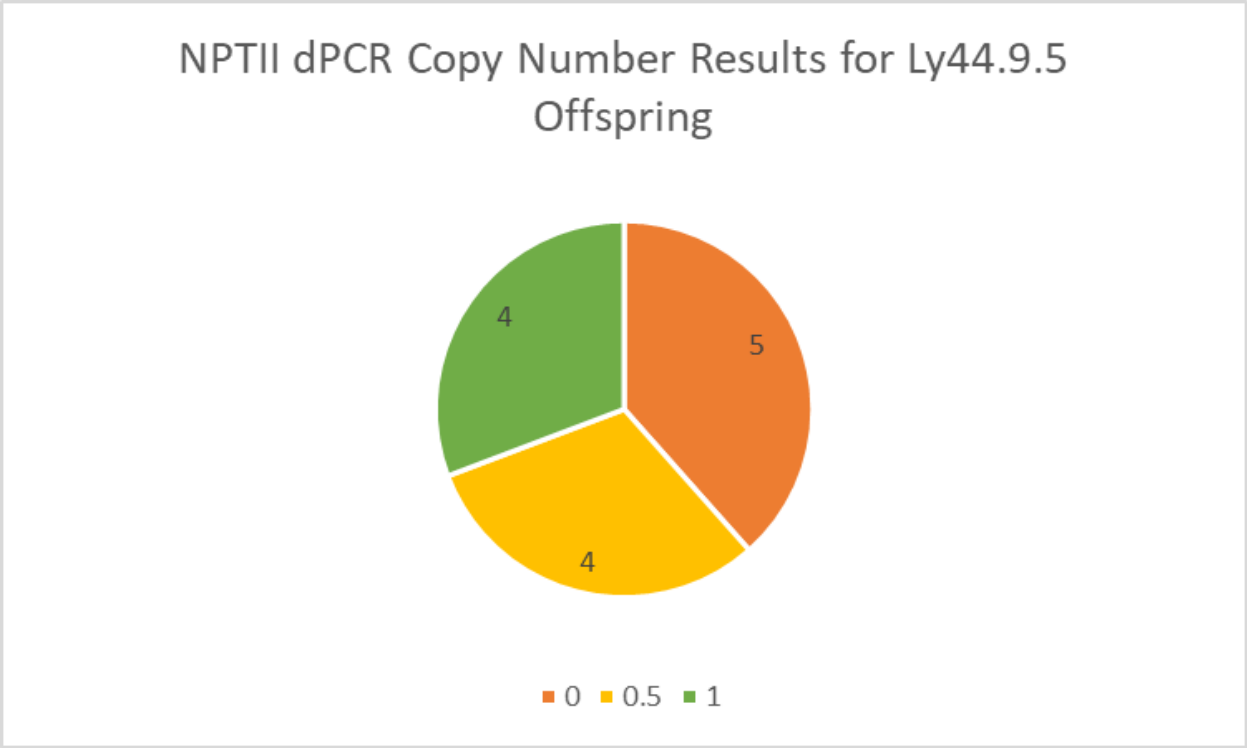


Figure 6. NPTII dPCR copy number results for Ly44.9.5 offspring.

Based on Mendelian Genetics, it was predicted that 25% of the plants would have a copy number of 0, 25% of the plants would have a copy number of 0.5, and 25% of the plants would have a copy number of 1.

Based on these figures, it is seen that the offspring of the plant Ly44.9.5 are not exact, but are close to following the expected percentages based on Mendelian genetics. For instance, 5 of these 13 plants are Tas14-REP negative, a 38% negative rate that was hypothesized to be 25%. For the NPTII dPCR, the expectation was that 25% of the plants would have a copy number of 0, 50% of the plants would have a copy number of 0.5, and 25% of the plants would have a copy number of 1. These percentages turned out to be 38, 31, and 31 respectively.

Although these predictions might seem far off, it is important to note that this was a small scale test. If, for instance, 100 plants had been tested, the law of large numbers would dictate

that we would have seen a Tas14-REP PCR negative vs positive distribution closer to 25% to 75%, and an NPTII dPCR copy number distribution closer to 25% to 50% to 25% for copy numbers of 0, 0.5, and 1 respectively. These percentages will therefore continue to be hypothesized as offspring are continually planted and made in the laboratory.

The digital drop PCR (dPCR) results for these plants were also included in the data. dPCR was conducted for the NPTII gene, the gene that should confer resistance to Kanamycin. Based on a plant with a known NPTII copy number, dPCR was able to determine the copy number of each plant tested. Theoretically, the NPTII gene, Tas14 promoter, and REP gene were inserted as a single unit of the T-DNA. However, it is possible to lose the Tas14 and REP genes during the transformation process, making it necessary to perform a PCR for the Tas14:REP junction as well. Table 2, which includes the most recent rounds of Tas14-REP PCR and NPTII dPCR, as well as those results obtained over the past few years, contains all of the currently known results for plants which there is both Tas14-REP PCR data and NPTII dPCR data.

Table 2. REP PCR and NPTII dPCR results for all REP plants in the laboratory

Plant Construct	REP PCR (+ or -)	dPCR Copy Number	Plant Construct	REP PCR (+ or -)	dPCR Copy Number	Key
Ly44.4	-	0.5	**Ly44.9.5.17**	-	0	REP PCR
Ly44.6	+	0.5	**Ly44.9.5.18**	+	0.5	-
Ly44.6.2	+	0.5	**Ly44.9.5.19**	+	1	+
Ly44.6.3	+	1	**Ly44.9.5.20**	+	0.5	
Ly44.7	-	1.5	**Ly44.9.8**	+	3	NPTII dPCR
Ly44.8	+	0.5	**Ly44.9.10**	-	0	0
Ly44.8.2.1	-	0	**Ly44.9.11**	+	0.5	0.5
Ly44.8.2.2	-	0	**Ly44.9.12**	+	0.5	1
Ly44.8.2.3	-	0	**Ly44.9.13**	+	0.5	>1
Ly44.8.2.5	-	0	**Ly44.9.14**	+	0.5	
Ly44.8.2.6	+	0.5	**Ly44.9.15**	+	0.5	
Ly44.9.2	+	3	**Ly45.6.1**	+	1	
Ly44.9.5.5	+	1	**Ly45.6.2**	+	1	
Ly44.9.5.9	-	0	**Ly45.6.2.1**	+	1	
Ly44.9.5.10	-	0	**Ly45.6.2.2**	+	1	
Ly44.9.5.11	+	0.5	**Ly45.6.2.3**	+	1	
Ly44.9.5.12	+	0	**Ly45.6.4**	+	1	
Ly44.9.5.13	+	0.5	**Ly45.6.4.1**	+	1	
Ly44.9.5.14	+	1	**Ly45.6.4.3**	+	1	
Ly44.9.5.15	-	0	**Ly45.9**	-	1	
Ly44.9.5.16	-	1				

The major conclusion that can be taken from this table is what must be done with the plant next in order to produce a homozygous line of REP+ plants. Any of these plants with a copy number of 1 and a parent copy number of 0.5 are at presumed to be homozygous, because the gene insertion must be the same on both chromosomes. However, plants with a copy number of 1 but a parent copy number of anything other than 0.5 are not as useful, as it cannot be certain whether it is two separate gene insertions in different parts of the chromosome, or the gene insertion on the same spot in both chromosomes. Plants with a copy number of 0.5 are also valuable as those are the plants which offspring will be grown from with the intent to produce plants with a copy number of 1.

In a few cases, results in this table are not consistent with hypotheses. For instance, there are some plants with a negative Tas14-REP PCR result that have an NPTII dPCR copy number of 0.5 or higher, such as the construct Ly44.9.5.16. There are two possible explanations: (1) a failure of the PCR method, or (2) the Tas14-REP junction was dropped during transformation, but the NPTII gene was kept. The loss of the gene or selectable marker is rare but has been known to occur. For this reason, the failure of execution of the PCR was decided to be inadequate basis for the conclusion of loss of the REP gene unless there is an internal control for the extraction as noted in the improved method of inclusion of PCR of a native Systemin gene. Another example of results that do not support the hypotheses occur when a plant has a positive Tas14:REP result but an NPTII dPCR copy number of 0, such as the construct Ly44.9.5.12. These results are a bit harder to explain, and can most likely be attributed to experimental error. Notably, the presence of the kanamycin resistance gene (NptII) can also be included as an internal control for the PCR screening as well. Although the lab has moved

forward with PCR screening that has included these improvements, those methodologies are not reflected in the work of this thesis.

Future Work for REP+ Plant Screening

The ultimate goal of this stage of the process is to confirm homozygous Tas14:REP positive tomato plants. Ideally, this would be accomplished for all three truncations of the Tas14 promoter. This thesis work only dealt with the screening of the Ly44 and Ly45 lines. At this point, 17 T0 plants have been transformed for the Ly44 line, which produced 71 T1 plants, and 54 T2 plants. The Ly45 line had 17 plants originally transformed, producing 17 T1 plants, and 10 T2 plants.

From this work, multiple homozygous Ly44 plants appear to have been obtained from hemizygous parents. These include Ly44.6.3, Ly44.9.5.5, Ly44.9.5.14, and Ly44.9.5.19. However, the homozygous state is not confirmed until the subsequent segregation confirms that all offspring are the same (hence homozygous). These putative homozygous plants must be harvested for fruit and seeds. The seeds must then be planted, grown, and tested using the same procedures, to verify that all the offspring are positive for the for the Tas14:REP junction. The best possible confirmation, would then utilize digital PCR to show that the transgene copy number is same as the same single gene copy of the systemin gene (dPCR ratio = 1).

With regard to the Ly45 plants, on the 1000 base Tas14 promoter, hemizygous plants are still being sought. The current plants, all of which have a copy number of 1, must continue to produce offspring, until a hemizygous plant can be obtained. From here, the hemizygous plant's

seeds must be grown to test the next generation and produce a homozygous Tas14-REP positive line of Ly45 plants for future experimentation.

Chapter 6

Conclusions

In the end, this project is a step closer to the final experimentation stage. It has been shown that in planting a hemizygous seed, offspring can either have no copies of the gene, a copy of the gene on one chromosome, or a copy of the gene on both chromosomes. This modeling will prove useful as a line of NPTII homozygous, Tas14-REP positive plants are created, grown, maintained, and experimented upon. From a broader perspective, this project, along with the development of nanoparticle delivery discussed in Appendix B, will ultimately fit together within the larger project: complementation of deconstructed viral vectors by plants that are homozygous for the REP gene on the Tas14 promoter. Carbon nanoparticles will be used to test whether this viral complementation is a viable option before the experiment moves to a larger scale utilizing whitefly colonies. In the meantime, the most important aspect of the project is creating lines of these plants that are homozygous for the REP gene. It is vital that these plant lines are grown and maintained throughout the stages leading up to ultimate experimentation, so that they will be ready when needed.

Appendix A

Gels from Tas14-REP PCR

The following gels were performed between the summer of 2021 and the spring of 2022. They are based on what plants were viable and needed to be tested. There is no general structure other than chronology. The dates of each gel can be found in the PCR Diagnostic Gels and Legends document within the laboratory Drive folder.

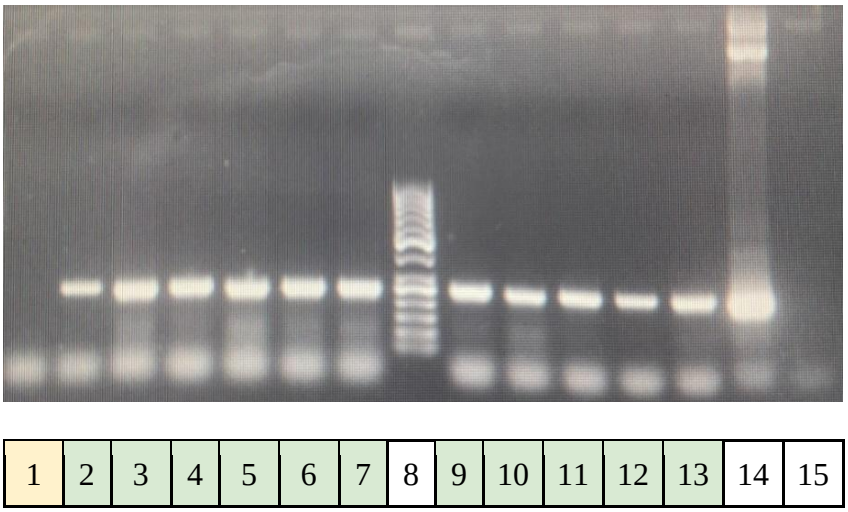
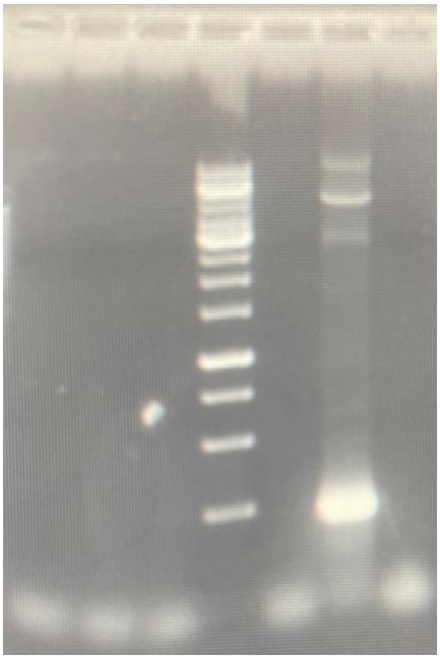


Figure 7. Gel 2

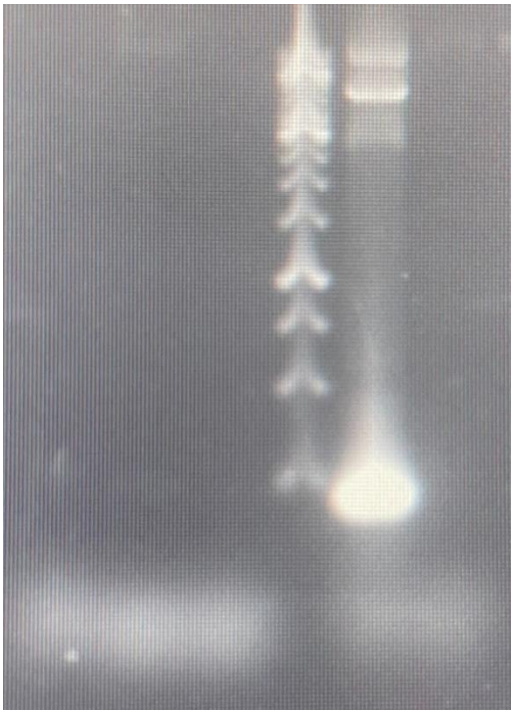
Lane 1: Ly44.8.2.5
Lane 2: Ly44.8.2.6
Lane 3: Ly45.6.2.1
Lane 4: Ly45.6.2.2
Lane 5: Ly45.6.2.3
Lane 6: Ly45.6.2.4
Lane 7: Ly45.6.2.5
Lane 8: 50 kb Ladder
Lane 9: Ly45.6.4.1
Lane 10: Ly45.6.4.2
Lane 11: Ly45.6.4.3
Lane 12: Ly45.6.4.4
Lane 13: Ly45.6.4.5
Lane 14: Positive Control
Lane 15: Negative Control



1	2	3	4	5	6	7

Lane 1: Ly44.8.2.1
Lane 2: Ly44.8.2.2
Lane 3: Ly44.8.2.3
Lane 4: 1 kb Ladder
Lane 5: Ly45.13b
Lane 6: Positive Control
Lane 7: Negative Control

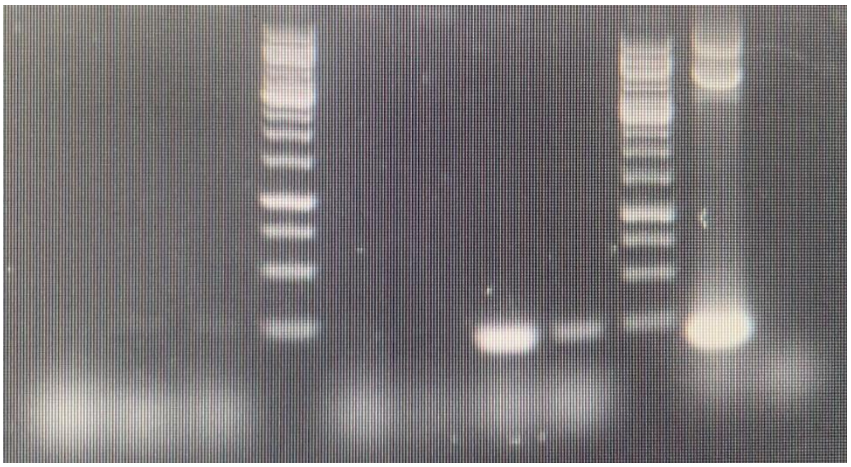
Figure 8. Gel 3



1	2	3	4	5	6

Lane 1: Ly44.8.2.1
Lane 2: Ly44.8.2.2
Lane 3: Ly44.8.2.3
Lane 4: 1 kb Ladder
Lane 5: Positive Control
Lane 6: Negative Control

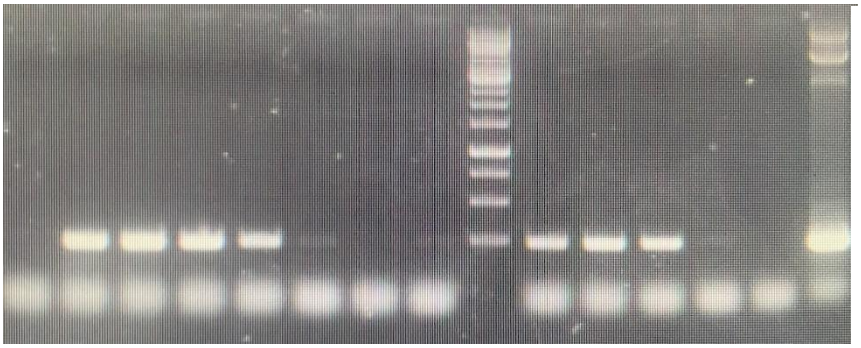
Figure 9. Gel 4



1	2	3	4	5	6	7	8	9	10	11

Lane 1: Ly44.8.5
Lane 2: Ly44.8.6
Lane 3: Ly44.8.7
Lane 4: 1 kb Ladder
Lane 5: Ly44.6.4
Lane 6: Ly44.9.5.4
Lane 7: Ly44.9.8
Lane 8: Ly45.13b
Lane 9: 1 kb Ladder
Lane 10: Positive Control
Lane 11: Negative Control

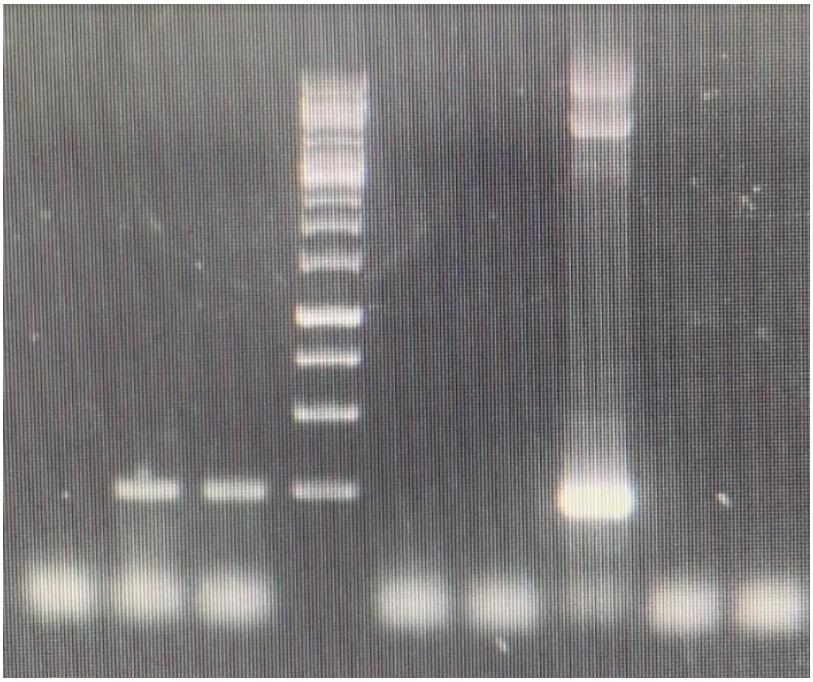
Figure 10. Gel 5



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Figure 11. Gel 6

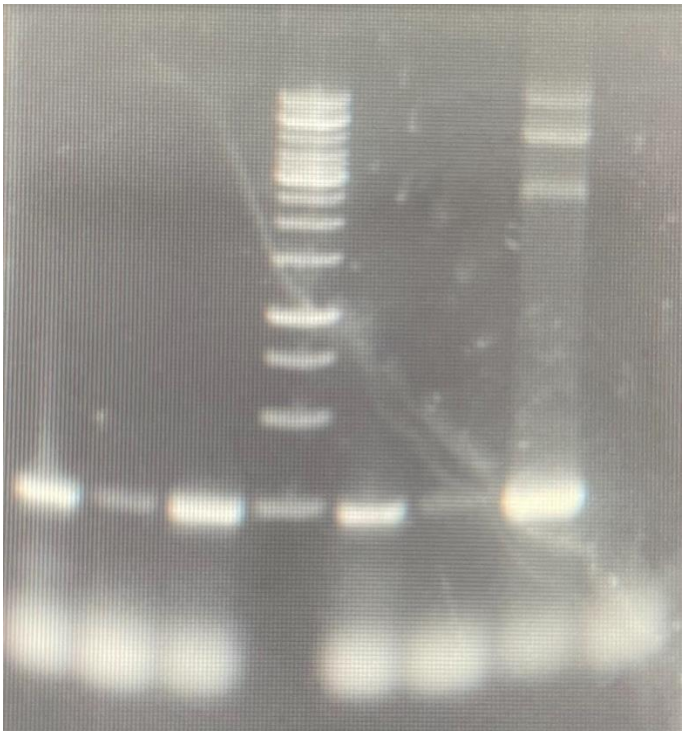
Lane 1: Ly44.9.5.4
Lane 2: Ly44.9.5.5
Lane 3: Ly44.9.5.6
Lane 4: Ly44.9.5.7
Lane 5: Ly44.9.5.8
Lane 6: Ly44.8.2.1
Lane 7: Ly44.8.2.2
Lane 8: Ly44.8.2.3
Lane 9: 1 kb Ladder
Lane 10: Ly44.6.1
Lane 11: Ly44.6.2
Lane 12: Ly44.6.3
Lane 13: Ly44.6.4
Lane 14: Positive Control
Lane 15: Negative Control



1	2	3	4	5	6	7	8	9

Figure 12. Gel 7

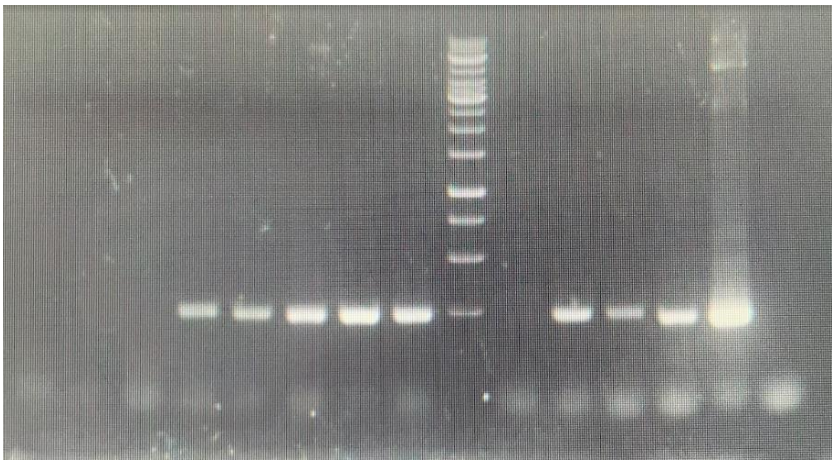
Lane 1: Ly44.7
Lane 2: Ly44.8
Lane 3: Ly44.10
Lane 4: 1 kb Ladder
Lane 5: Ly45.9
Lane 6: Ly44.4
Lane 7: Positive Control
Lane 8: Negative Control
Lane 9: Ly46.3



1	2	3	4	5	6	7	8

Lane 1: Ly45.6.6b2
Lane 2: Ly45.13b
Lane 3: Ly44.10b2
Lane 4: 1 kb Ladder
Lane 5: Ly44.3
Lane 6: Ly44.6
Lane 7: Positive Control
Lane 8: Negative Control

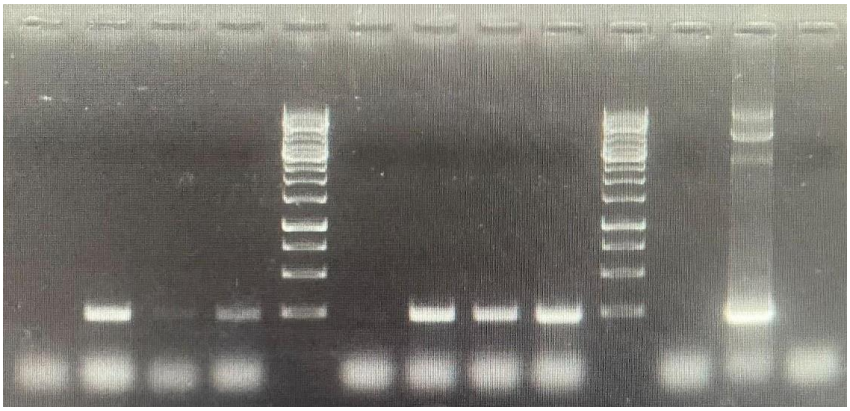
Figure 13. Gel 8



1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Lane 1: Ly44.9.3
Lane 2: Ly44.9.8
Lane 3: Ly44.9.10
Lane 4: Ly44.9.11
Lane 5: Ly44.9.13
Lane 6: Ly45.6.1
Lane 7: Ly45.6.2
Lane 8: Ly45.6.4
Lane 9: 1 kb Ladder
Lane 10: Ly45.9.1
Lane 11: Ly44.8b1
Lane 12: Ly44.8b2
Lane 13: Ly44.9.2
Lane 14: Positive Control
Lane 15: Negative Control

Figure 14. Gel 9



1	2	3	4	5	6	7	8	9	10	11	12	13

Lane 1: Ly44.9.9
Lane 2: Ly44.9.12
Lane 3: Ly44.8b2
Lane 4: Ly44.10b2
Lane 5: 1 kb Ladder
Lane 6: Ly45.6.3b1
Lane 7: Ly45.6.4
Lane 8: Ly44.9.14
Lane 9: Ly44.9.15
Lane 10: 1 kb Ladder
Lane 11: Ly44.4
Lane 12: Positive Control
Lane 13: Negative Control

Figure 15. Gel 10

Appendix B

Nanoparticle Work and Future Plans

Introduction

During the experimental stages of this project, the goal is to use nanoparticles to deliver the Tomato Mottle Virus. Nanoparticles tend to achieve successful delivery of smaller vectors, so the minimal cloning vector, pICOz, was obtained. Insertion of the virus into the minimal cloning vector has proved challenging, but future work will allow final nanoparticle delivery of the deconstructed viral vector.

Background

Although Agrobacterium transformation has been and continues to be the most commonly used method to introduce foreign genes into plant cells, there are some disadvantages, such as low transformation efficiencies, unavoidable damage to plant tissues, and limits as to which plants will accept that DNA. Another issue is that Agrobacterium tends to modify the genome of the plant, which can be useful if that's what is being attempted, but can be a negative side effect if the trait being introduced is meant for just that plant and not its offspring. Because of these issues, new technologies have been developed. One such technology is known as biolistic particle delivery, or gene gun, and has had success in increasing the host range, but also only causes expression of the gene at the bombardment site and can cause tissue damage as well.

Recently, scientists have begun exploring the use of carbon nanoparticles as a DNA delivery vector. For instance, technologies such as mesoporous silica nanoparticles (MSNs),

DNA nanostructures, DNA origami, silicon carbide whiskers (SCWs), and layered double hydroxide clay nanosheets have all shown promise in DNA delivery to plant cells. For instance, plasmid DNA has been introduced into Arabidopsis by MSN's, and LDH's have delivered RNA that has resulted in gene silencing in Nicotiana tabacum species.[21]

The ultimate goal of DNA delivery via nanoparticles is to deliver plasmid DNA to plant cells at high efficiencies without transgene integration of the plant species. Carbon nanotubes (CNTs) have been seen to do just this, getting by the chloroplasts and plant membranes by passive transport. Their ability to do this is likely due to their large aspect ratio, biocompatibility, and high tensile strength. They are also well below the maximum size to traverse the plant cell wall. While being transported, CNTs protect biomolecules from being degraded or metabolised in the processing.[21]

The Minimal Cloning Vector pICOz

In this lab, the plasmid pLSU has been used as a backbone where viruses or other DNA sequences can be inserted and delivered to Agrobacterium for ultimate plant transformation. Although this works well for Agrobacterium, grafting DNA onto carbon nanotubes becomes challenging with longer sequences of DNA. Because of this, it is necessary to find the shortest vector backbone possible to deliver DNA via nanoparticles.

In nature, the smallest plasmid observed was 746 bp. To come as close to that number as possible, the minimal cloning vector pICOz was created from the original plasmid pUC18 by Dr. Jens Staal and subsequently obtained by CurtisLab. pCIOz is 1185 bp in length, with a fully functional multiple cloning site and zeocin resistance, which makes pICOz very practical because E. *coli* is not naturally zeocin resistant.[22,23]

Experiments and Hypotheses

In order to introduce the viral DNA to the plant, a vector must be used. The final goal is to use whiteflies, but with regulations, use of a BSL3 lab is required. To save time and money in the earlier stages of the project, and to be able to do as much proof of principle testing as possible, some other method must be used to introduce the DNA. Because Agrobacterium can express the ToMoV virus by itself, it is difficult to determine whether or not the virus has been taken up and expressed in the plant. This leaves carbon nanotubes as the next viable option. Carbon nanotubes are more successful at delivering smaller sequences of DNA, so the ToMoV virus must be taken out of its original pLSU backbone and inserted into the pICOz backbone obtained from Dr. Jens Staal.[23]

The idea that carbon nanotubes can deliver viral DNA at all will first be tested with the wild type ToMoV virus on Nicotiana *benthamiana*, a model species when it comes to introducing DNA. If successful, the same process will then be performed using the deconstructed ToMoV vector on the Lycopersicum plants that have the genes for the ToMoV REP and CP on the Tas14 promoter. Drought or some other stress will be mimicked, expressing the REP and CP, and hopefully complementing the ToMoV, expressing the virus in the plant, all while being incapable of transfer to other plants. If this is successful, it proves that plants can complement deconstructed viral vectors, setting the stage for the beneficial viruses to be introduced by nanoparticles and ultimately whiteflies.

Materials and Methods

pICOz Preparation

The pICOz plasmid was shipped and received in solid, powder form. This pICOz powder was subsequently resuspended and stored in TE buffer.

LB+Zeocin25 Media Preparation

The recipe for LB media is shown in Table 3 below. Zeocin was also added at a concentration of 25 ug/mL.

Table 3. Solid LB media recipe

	Final Concentration (g/L)
Bacto Tryptone	10
Yeast Extract	5
NaCl	10
Agar (for solid media)	10
Final Target pH	5.7

pICOz cultures were then grown on an LB+Zeocin25 plate. One of these cultures was chosen from the plate, and subsequently allowed to grow for 18 hours in LB+Zeocin25 liquid media. After 18 hours of growth a plasmid preparation procedure from IBI Scientific[24] was used to obtain the pICOz plasmid.

Digestion of the Tomato Mottle Virus B

A digestion reaction was performed to remove the ToMoVB virus from the pLSU backbone. For this reaction, reagents were added in the amounts seen in Table 4, and the solution was incubated overnight at 37 °C.

Table 4. ToMoVB digestion solution

ToMoVB Sample (250 ng/uL)	2.5 uL
SphI	1 uL
XhoI	1 uL
10x CutSmart Buffer	2 uL
DEPC Water	13.5 uL

Digestion of the Tomato Mottle Virus A

To create the correct restriction sites, a PCR was run on the ToMoVA sequence. The primers used added the restriction site KpnI. This site, along with a blunt end site, were necessary for the digestion and ligation in the correct orientation. The PCR results were then run on a gel, recovered, and subsequently ligated into pJet, a plasmid backbone that does not require the sticky ends to match up. This pJet plasmid was then inserted into NEB Stable E. *coli* using heat shock transformation. This procedure involved combining the plasmid solution with the E. *coli* and placing this combined solution in a water bath at 42 °C for 45 seconds, then growing colonies on LB+Zeocin25 plates for 24 hours. Colonies were taken from these plates and grown in LB+Zeocin25 liquid media for 18 hours. The plasmid preparation procedure was then performed, and a digestion solution was made using the reagents in Table 5.

Table 5. ToMoVA digestion solution

ToMoVA Sample (250 ng/uL)	2.5 uL
KpnI	1 uL
10x CutSmart Buffer	2 uL
DEPC Water	14.5 uL

Ligation of pICOz with ToMoVA and ToMoVB

To insert the respective TomovA and TomovB viruses into the pICOz plasmid backbone, the pICOz was first digested using the same respective restriction enzymes as the virus being inserted. The ligation solution was made using the reagents in Table 6 and allowed to react at 4 °C for 16 hours.

Table 6. Ligation solution

T4 DNA Ligase Buffer (10X)	2 uL
pICOz	1 uL
ToMoV (A or B)	1 uL
Nuclease-free water	15 uL
T4 DNA Ligase	1 uL

The pICOz plasmids were then inserted into NEB Stable *E. coli* using heat shock transformation, and grown on LB+Zeocin25 plates for 24 hours. Colonies were taken from the plates and grown for 18 hours in LB+Zeocin25 liquid media. Plasmid preparation was subsequently performed.

Nanoparticle Delivery Progress

The next major step in this project is to develop techniques to deliver the deconstructed viral vectors to the tomato plants via nanoparticle delivery. This process has begun, but as of this time is still in the nascent stages.

The minimal cloning vector was obtained from Dr. Jens Staal's laboratory. This minimal cloning vector, pICOz, was then transformed into Top10 *E. coli*. After being extracted again into plasmid form, digestion, ligation, and heat shock transformation were attempted between the pICOz plasmid and the ToMoVA and ToMoVB plasmids respectively.

For ToMoVA, this process included adding restriction sites via PCR so that the virus would insert into the pICOz plasmid in the correct orientation. One site added was a KpnI restriction site, and the other end was attempted via blunt end cloning. Once a PCR and gel were performed, adding these restriction sites, an extraction was performed for the new virus strand. This was then ligated into pJet and transformed into a Top10 E. *coli* colony. Finally, the pJet plasmid containing the ToMoVA virus was again extracted and digested with KpnI. pICOz was also digested with KpnI. The ligation technique was then attempted on these two digestion solutions. At this current point, there have been no successful ligations between ToMoVA and pICOz.

For ToMoVB, the process was simpler as digestion using restriction sites in the ToMoVB plasmid would create the correct orientation within the pICOz plasmid. Therefore, solutions of both were digested using HindIII and KpnI, and a ligation attempt between these solutions was subsequently attempted However, at this time there have been no successful ligations between ToMoVB and pICOz.

Once both ToMoVA and ToMoVB can be successfully ligated into the minimal cloning vector pICOz, the process of grafting these plasmids to the carbon nanoparticles can be attempted, with the ultimate goal of obtaining successful delivery and expression of the virus within Nicotiana benthamiana plants.

Conclusions

The nanoparticle delivery experiments are still in the nascent phases. Before any experiments can be run, both the A and B components of the Tomato Mottle Virus must be inserted into the minimal cloning vector pICOz. Once this occurs, the process of grafting the viral plasmids onto carbon nanoparticles can be performed. Subsequent plant infiltration of these nanoparticle-viral complexes will be performed with the goal of viral expression within the plant.

Appendix C

pICOz Seuqence

```
   1 acgcgtcgcg aggccatatg ggttaaccca tggccaagct tgcatgcctg caggtcgact
  61 ctagaggatc ccgggtaccg agctcgaatt cggatatcct cgagacagtg ggcccgttta
 121 aacacatgtg tttttccata ggctccgccc ccctgacgag catcacaaaa atcgacgctc
 181 aagtcagagg tggcgaaacc cgacaggact ataaagatac caggcgtttc cccctggaag
 241 ctccctcgtg cgctctcctg ttccgaccct gccgcttacc ggatacctgt ccgcctttct
 301 cccttcggga agcgtggcgc tttctcatag ctcacgctgt aggtatctca gttcggtgta
 361 ggtcgttcgc tccaagctgg gctgtgtgca cgaaccccc gttcagcccg accgctgcgc
 421 cttatccggt aactatcgtc ttgagtccaa cccggtaaga cacgacttat cgccactggc
 481 agcagccact ggtaacagga ttagcagagc gaggtatgta ggcggtgcta cagagttctt
 541 gaagtggtgg cctaactacg gctacactag aagaacagta tttggtatcc gcgctctgct
 601 gaagccagtt accttcggaa aaagagttgg tagctcttga tccggcaaac aaaccaccgc
 661 tggtagcggt ggtttttttg tttgcaagca gcagattacg cgcaggaaaa aaggatctca
 721 agaagatcct ttgatctttt ctacgtcagt cctgctcctc ggccacgaag tgcacgcagt
 781 tgccggccgg gtcgcgcagg gcgaactccc gcccccacgg ctgctcgccg atctcggtca
 841 tggccggccc ggaggcgtcc cggaagttcg tggacacgac ctccgaccac tcggcgtaca
 901 gctcgtccag gccgcgcacc cacacccagg ccagggtgtt gtccggcacc acctggtcct
 961 ggaccgcgct gatgaacagg gtcacgtcgt cccggaccac accggcgaag tcgtcctcca
1021 cgaagtcccg ggagaacccg agccggtcgg tccagaactc gaccgctccg gcgacgtcgc
1081 gcgcggtgag caccggaacg gcactggtca acttggccat actcttcctt ttcaatatta
1141 ttgaagcatt tatcagggtt attgtctcat gagcggatac ata
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Figure 16. pICOz Sequence[25]

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ACADEMIC VITA

EDUCATION

The Pennsylvania State University, University Park, PA
Schreyer Honors College Scholar — May 2022
Bachelor of Science in Chemical Engineering

WORK EXPERIENCE

Undergraduate Research, *CurtisLab* — August 2020-Present
University Park, PA

- Develop techniques for nanoparticle delivery of viral DNA for gene editing purposes
- Manipulate viruses to optimize expression and alter plant stress response pathway
- Genetically transform plants for compatibility with specific deconstructed viral vectors
- Troubleshoot and modify standard operating procedures when issues arise
- Communicate results to other students and researchers via presentations and papers

Research and Development Lab Technician, *ANKOM Technology* — July 2020-August 2020
Macedon, NY

- Performed testing procedures on ANKOM Flex during development process
- Carried out chemical processes that extract macromolecules from food sources
- Altered these processes to make machines more efficient and cost effective
- Analyzed test results and communicated needed alterations based on these results

Program Manager, *Church of the Saviour* — May 2019-August 2019
Wayne, PA

- Planned and executed the program for 3 summer camps
- Managed hundreds of counselors and volunteers
- Received training in leadership development

LEADERSHIP & INVOLVEMENT

Small Group Leader, Vice President, Treasurer; Penn State Navigators	2018-Present
Social Media Chair; Penn State Outing Club	2018-2020
Member; Penn State Spikeball Club	2018-Present
Member; Soccer, Basketball, Frisbee, Hockey, Table Tennis, Volleyball Intramurals	2018-Present
Influencer; Instagram (Account Username national_park_photography)	2014-Present

COMMUNITY SERVICE

Citizens Corps of Delaware County COVID testing volunteer	2020
Spring Break Service Trips to Philadelphia, North Carolina	2019, 2020
Summer Camp Counselor	2013-2020
YWAM Service Trip to Belo Horizonte, Brazil	2018

AWARDS AND SCHOLARSHIPS

Academic Excellence, Klinger Chemical Engineering, and CSL Behring Biotechnology Scholarships	2018-Present
REU Biofellowship	2021
Best Engineered Design, College of Engineering Design Showcase	2019