

April 2008

Construction Of A Turnip Crinkle Virus Mutant

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CONSTRUCTION OF A TURNIP CRINKLE VIRUS MUTANT

A Major Qualifying Report:

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

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Date: April 24, 2008

Approved:

Professor Kristin Wobbe, Major Advisor

Abstract:

An important immune response to pathogens in *A. thaliana* and other plants is the hypersensitive response (HR). A HR is a form of programmed cell death that prevents the spread of an infection by necrosis of tissue at the site of infection. Turnip Crinkle Virus (TCV) is unique in the Carnovirus genus for its ability to suppress the HR in systemic infection of *A. thaliana*. It is hypothesized that the p8 viral movement protein of TCV is responsible for this ability, due to that protein's nuclear localization. It should be possible to test if p8 suppresses the HR by replacing it with a homologous gene. Genetic modifications were made to an expression vector containing a dsDNA copy of the TCV ssRNA genome, pT1D1ΔL. The viral genome was altered to eliminate expression of p8. The inserted homologous gene was p7, from the related Carnation Mottle Virus. Single-nucleotide substitution was done with PCR to remove the start codon of p8. A DNA cassette based on the p7 gene was constructed from oligonucleotides. The cassette was the template for insertion of p7 into the vector. Recombination of the p7 gene and the expression vector was accomplished with PCR and New England Biolab's USER enzyme.

Acknowledgments:

I would like to thank Professor Kristin Wobbe for giving me the opportunity to work on this project, and for her constant advice and encouragement.

I would also like to thank Patrick Arsenault, whose instructions for experimental techniques were invaluable, for assistance with resources and supplies, and for always answering my questions that came up in the laboratory.

Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
Table of Figures	v
1. Introduction	1
2. Materials and Methods	9
3. Results	34
4. Discussion	48
5. References	52

Table of Figures:

1.1 Gene map of TCV	1
1.2 CarMV gene map.....	6
2.1 Photo of <i>Nicotiana benthamiana</i> in growth chamber	13
2.2 Photo of <i>Arabidopsis thaliana</i>	18
3.1 PCR single-nucleotide substitution.....	35
3.2 Map of p7-insert cassette	37
3.3 Recombination of the PCR products with USER enzyme reaction.....	38
3.4 Gene map of mutant TCV transcribed from pTCVNF1	39
3.5 Gel electrophoresis of PCR single-nucleotide substitution	39
3.6 Gel electrophoresis of p7 cassette PCR	41
3.7 Gel electrophoresis of pT1D1ΔLΔp8 PCR.....	42
3.8 Gel electrophoresis of Taq PCR screening of colonies	43
3.9 Gel electrophoresis of diagnostic Taq PCR of insert.....	44
3.10 Gel electrophoresis of restriction digests of plasmids	45
3.11 Agarose gel electrophoresis of viral RNA transcripts	47

1. Introduction

1.1 Turnip crinkle virus

Turnip crinkle virus (TCV) is a plant virus of the genus Carmovirus, in the family Tombusviridae. TCV has a single-stranded positive-sense RNA genome. The genome codes for 5 proteins: 2 RNA-dependent RNA polymerases (RdRp), 2 movement proteins (MP), and a coat protein (CP) (Hacker et al., 1992). The RdRp proteins are p88 and p28. P88 is a read-through product of the open reading frame of p28. P8 and p9 are the movement proteins. All of the coding sequences of TCV overlap with adjacent coding sequences. This genome arrangement is representative of all viruses in Carmovirus (Weng and Xiong, 1997).

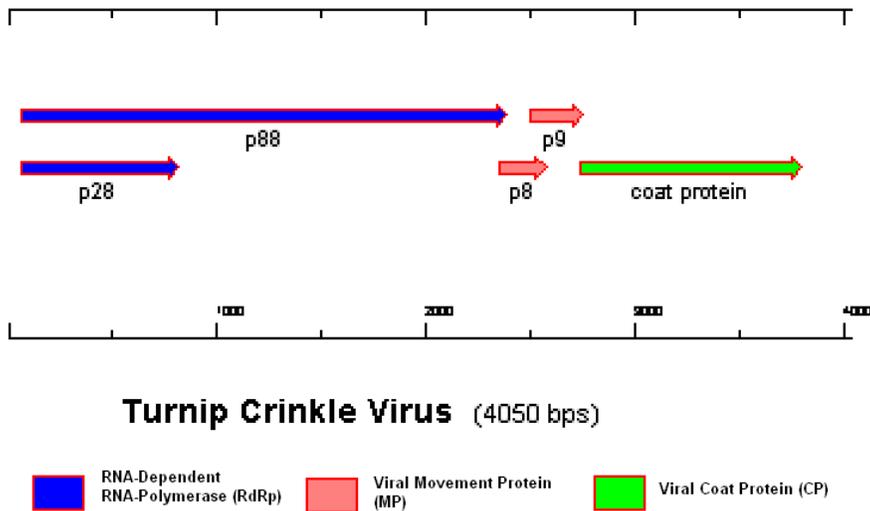


Figure 1.1: Gene map of TCV, created with Clone Manager 7, from GenBank file NC_003821. p9 gene location added based on Hacker et al.

When TCV infects a plant, p88 and p28 are translated by host ribosomes from the whole genome strand. P88 is translated due to p28 stop codon read-through. Replication

of the genome is accomplished by synthesis of a negative-sense RNA copy of the genome for use as a template. Although only P88 contains a known RdRp motif, it is known that both proteins are required for replication of the genome in viruses of the related Tombusvirus genus (Hull, p. 331). The other genes of TCV are translated using a combination of subgenomic RNAs (sgRNA) and “leaky scanning.”

Two sgRNAs are made, and are both co-terminal with the 3’ end of the genome. A 1.7 kb sgRNA encodes p8, p9, and the coat protein gene. A 1.45 kb sgRNA contains the coding sequence for only the coat protein gene, and is the only RNA from which the gene is translated. Promoters for synthesis of the sgRNAs are found within the regions of the negative-sense whole genome template complementary to p88 and p9 for the 1.7 kb sgRNA and 1.45 kb sgRNA, respectively (Wang & Simon, 1997).

The movement proteins are both translated from the 1.7 kb sgRNA, using leaky scanning (Li et al., 1998). Leaky scanning is a polycistronic translation strategy used by some RNA viruses. A sub-optimal translation initiation site, one that is dissimilar from the Kozak consensus sequence, causes the 40S ribosome subunit to infrequently fail to initiate at the most 5’ start codon, instead scanning to a downstream start codon (Kozak, 2002).

1.2 Turnip Crinkle Virus Movement Proteins

Plant viruses spread through plants in two ways. In cell-to-cell movement, the virus spreads to cells contiguous to the infected cell by passing through plasmodesmata. Plasmodesmata are pores in plant cell walls that provide intercellular connections for the cytoplasm and endoplasmic reticulum. In systemic movement, the virulent particles enter

the phloem and are spread to plant cells away from the site of infection (Hull, pp. 395-400).

P8 and p9 are both necessary for cell-to-cell and systemic movement of TCV (Hacker et al., 1992). The proteins function by *in trans* complementation, with p8 more abundant than p9 (Li et al., 1998). P8 was found by yeast two-hybrid studies to bind an *A. thaliana* protein designated Atp8 (Lin & Heaton, 2001). Sequencing of Atp8 indicated that the protein contains two possible transmembrane helices.

Two regions of p8 bind RNA (Akgoz et al., 2001). The protein also has two nuclear localization signals (NLS), and localizes to the plant cell nucleus of *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves in green fluorescent protein (GFP) fusion studies (Cohen et al., 2000). Combined mutations of both NLS eliminated localization to the nucleus. This nuclear localization function has not been observed in any other Carmovirus movement protein. P9-GFP fusion protein was found in the cytoplasm and nucleus.

The function of p8 and p9 in viral movement has not been well-characterized. Wobbe et al. (1998) found that a single amino acid difference in p8 between TCV strain B and strain M was responsible for greater virulence and RNA binding ability in TCV-B. Possible functions of p8 and p9 may be inferred from the known functions of the movement proteins of other ssRNA plant viruses. The movement protein of *Tobacco mosaic virus*, p30, has a number of interactions with the plant and the single-stranded RNA viral genome (Hull, pp. 381-382). P30 is phosphorylated by at least one plant protein. This phosphorylation is required for transport of the virus through plasmodesmata. P30 localizes to the plasmodesmata, and increases the size exclusion

limit of the plasmodesmata to help the viral genome pass through. P30 also localizes to microtubules of the plant cell. P30 binds to nucleic acids by melting the secondary structure of the single strand. Pectin methylesterase (PME) from tobacco plants interacts with p30, and deletion mutagenesis showed the PME binding domain was necessary for cell-to-cell movement. This suggests that a primary function of RNA plant virus movement proteins is to transport viral RNA to plasmodesmata and enable passage through the pore.

1.3 Hypersensitive Response in *Arabidopsis thaliana*

Arabidopsis thaliana is a host of TCV. Inoculation of TCV virions into leaves of *A. thaliana* strains Di-0 or Di-17 will cause necrosis at the site of infection, due to a plant defense mechanism known as the hypersensitive response (HR). The necrosis is caused by programmed cell death (apoptosis) and is thought to be a means to stop spread of the virus. The guard hypothesis for plant immune defense proposes that two plant proteins and one viral gene product are required for triggering plant immune responses to a specific virus. A specific viral gene product, termed an *avr*, forms a complex with a plant protein. This complex is recognized by an R protein. The R protein then initiates a defense response in the cell. In *A. thaliana*, the R protein is HRT. In the presence of the TCV *avr*, the coat protein, HRT induces hypersensitive response along with a signal transduction cascade involving salicylic acid and other compounds. (Dempsey et al., 1997) Systemic necrosis from the hypersensitive response can occur as a result of viral infection, usually as a result of infected lesions that encompass veins (Hull, p. 450).

The amino terminus of the TCV coat protein contains the site responsible for plant recognition of the protein (Zhao et al., 2000). Ren et al. (2000) discovered that a protein in *A. thaliana* named TIP binds to the coat protein of TCV. Mutations that prevent the binding of TIP and the coat protein result in the loss of HR against TCV in inoculated plants. TIP may be a transcriptional activator of plant defense, and the TIP-coat protein complex may be recognized by HRT as the signal to begin HR.

1.4 TCV Systemic Infection and Suppression of the Hypersensitive Response

In systemic infection by plant viruses, spread of the virus occurs mostly through the phloem instead of cell-to-cell movement of the virus (Hull, pp. 397-400). Viruses are present in the greatest density in maturing leaves throughout the plant, following a source-to-sink transmission pattern, similar to the flow of sugars and other nutrients in the phloem (Hull, pp. 404-406). Dempsey et al. (1993) found that systemic infection in the Di-3 strain of *A. thaliana* gave symptoms in which new leaves were striped yellow and dark-green, followed by crinkling of new leaves and stunting of bolts. This led to death of the plant 18-21 days post-inoculation. Hypersensitive response was not observed in Di-3 in response to local or systemic infection. Some plants of the Di-17 strain of *A. thaliana* developed systemic infection. After one week, about 5% of the inoculated plants developed symptoms similar to those of the Di-3 strain. After two weeks, about 25% of the plants had systematic symptoms of drooping bolt tips and curled siliques.

In the Di-17 strain of *A. thaliana*, HR was suppressed in systemic TCV infection (Hammond, 2001). Initial inoculation of leaves caused necrosis, but other infected leaves had no necrosis. Symptomatic leaves showed chlorosis, and microscopic examination of

the leaves showed signs of necrotic cell death only in vasculature at the base of symptomatic leaves. Furthermore, the introduction of other infectious agents that normally induce HR in *A. thaliana* did not cause HR in plants with systemic infection of TCV.

1.5 Carnation mottle virus p7 Movement Protein

Carnation mottle virus (CarMV) is the type species of the Carmovirus genus. Its genome is organized similarly to TCV.

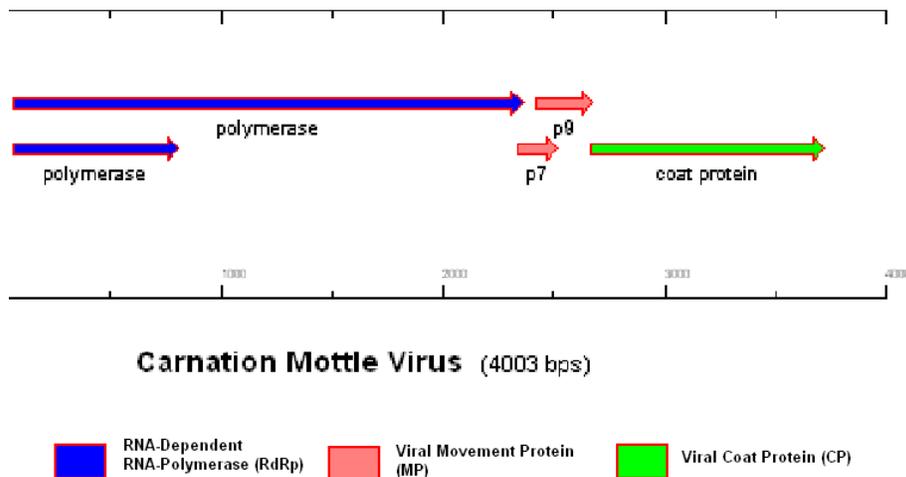


Figure 1.2: CarMV gene map. Created with Clone Manager 7, from GenBank file NC_001265.

P7 is the homolog of *Turnip crinkle virus*' p8. It contains one known RNA binding domain (Vilar et al., 2001). It does not contain a NLS. The amino acid sequences of TCV P8 and CarMV P7 have 32.8% identity (Canizares et al., 2001). Cohen et al. (2000) found that p7 is not localized exclusively to the nucleus.

1.6 *Arabidopsis thaliana* as a Model Plant for Viral Infection

A. thaliana is a member of the mustard family. It is the model plant for many areas of molecular, biological, and genetic research. Its small size, prolific seed production, short reproductive cycle (6 weeks), and relatively small genome (7×10^7 bp) contribute to the versatility of use in the laboratory and large-scale experimentation (About *Arabidopsis*). The genome has been completely sequenced by the *Arabidopsis* Genome Initiative, and many mutant strains are widely available. Despite the lack of economic utility, *A. thaliana* is a useful model for plant genetics as it is a relative of turnip, broccoli, cabbage, and other crop plants (*Arabidopsis: The Model Plant*). Additionally, the small genome is due to *A. thaliana* not undergoing polyploidy from agricultural breeding. *A. thaliana* is a good system for studying plant pathogens, and was used by Hammond (2001) to study suppression of HR by TCV. However, propagation of large amounts of plant pathogens requires larger plants.

1.9 Project Goals

It is hypothesized that the nuclear localization of p8 is part of a mechanism for the suppression of HR. The goal of this project is to show that the nuclear localization is necessary for TCV to suppress the HR, by replacing p8 with p7 from CarMV. The movement protein from CarMV was chosen because it is a homolog to p8 from a close relative of TCV, but does not have a NLS or localize to the nucleus. It is hypothesized that CarMV p7 will restore movement of the virus after inactivation of p8, but not restore an ability to suppress the HR. PCR-based mutagenesis of a cDNA copy of the TCV genome is the best method for this. Due to the overlap of p8 and other genes, it will be

necessary to avoid changing codons in the ORF of p88 and p9. Also, leaky scanning from p7 to TCV p9 will have to be preserved to ensure translation of both proteins.

After completion of the modification of the TCV cDNA, RNA from the mutant and wild-type will be transcribed and inoculated into *A. thaliana* to observe the phenotype of infection in the mutant virus. Mutant TCV RNA will also be inoculated into *Nicotiana benthamiana* to create large quantities of virions.

2. Materials and Methods

2.1 pT1D1ΔL

A dsDNA copy of the TCV-B genome is inserted downstream of a T7 RNA polymerase promoter in pT1D1ΔL. After the 3' end of the TCV genome is an XbaI site. The plasmid contains an ampicillin resistance gene and a bacterial origin of replication. It was created by Wobbe by eliminating the remaining polylinker region of pT1D1. Electroporation-transformed DH5α *E. coli* were the source for pT1D1ΔL.

2.2 Water

All water used for solutions was distilled twice. Water was sterilized by autoclaving. RNase-free water was prepared by DEPC-treatment followed by neutralization of pH with concentrated NaOH or HCl, then autoclaved to sterilize.

2.3 Plasmid DNA Miniprep Solutions

Glucose-Tris-EDTA (GTE) Solution: 50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8.0. Autoclaved and stored at 4° C.

Alkaline Lysis Solution II: 0.2 M NaOH, 1% SDS. Prepared fresh from 2 N NaOH and 10% SDS.

Alkaline Lysis Solution III: 5 M potassium acetate, buffered with acetic acid to pH 4.0. Stored at 4° C.

TE buffer: 10 mM Tris-Cl, 1 mM EDTA, pH 7.5.

2.4 DNA Primers

NF TCV Positive	5'-GATAGGTGGATCCTGAACGAATTCC-3'
NF TCV Negative	5'-ACTAAGTTCTTCTTTATATCCCGTGGG-3'
TCV USER neg	5'-AGATCTAAUGCTAGCTACCAATCTCTTCT-3'
TCV USER pos	5'-ATTAGAAAUGAAGGTTCTGCTAGT-3'
NF p7 USER R	5'-ATTTCTAAUGACAACGCTATTATCTG-3'
NF p7 USER L	5'-ATTAGATCUCTCACCATCG-3'

“NF TCV Positive” and “NF TCV Negative” were ordered from Sigma (St. Louis, MO). “TCV USER neg,” “TCV USER pos,” “NF p7 USER R,” and “NF p7 USER L” were ordered from Invitrogen (Carlsbad, CA). All primers were stored in 100 μ M solutions in 1X TE buffer. 10 μ M solutions in 0.1X TE buffer were made for use in PCR.

2.5 Polymerase Chain Reaction

Sigma REDaccuTaq LA DNA polymerase and Stratagene (Cedar Creek, TX) PfuTurbo C_x DNA polymerase were used for genetic modification and DNA amplification. Sigma Taq DNA polymerase was used for diagnostic purposes. The reaction buffer supplied with each product was also used. All PCR reactions were done in 0.2 mL PCR tubes.

2.6 P7 DNA Cassette Oligonucleotides

P7 CST (-)8	5'-GGTGAGAGATCTAATGGG-3'
P7 CST (+)1	5'-CCCATTAGATCTCTCACCATCGACACCAATGTGGGG-3'
P7 CST (-)7	5'-CCGCTTCACACTGGGGCCCCACATTGGTGTCG-3'
P7 CST (+)2	5'-CCCCAGTGTGAAGCGGCAGATTCACTATGGATATTGAAC-3'
P7 CST (-)6	5'-CAACTACTGGTACTTCCGGTTCAATATCCATAGTGAATCTG-3'

P7 CST (+)3	5' -CGGAAGTACCAGTAGTTGAGAAGCAAATGCTCGC-3'
P7 CST (-)5	5' -TTTGT'TTTCTCTATTCCCAGCGAGCATTGCTTCT-3'
P7 CST (+)4	5' -TGGGAATAGAGGAAAACAAAAGACACGTAGATCGGTG-3'
P7 CST (-)4	5' -GGATGGCGTCCTTGGCCACCGATCTACGTGTCT-3'
P7 CST (+)5	5' -GCCAAGGACGCCATCCGCAAACCTGCATCCGA-3'
P7 CST (-)3	5' -ATTACCCCCGTTAGTACTATCGGATGCAGGTTTGC-3'
P7 CST (+)6	5' -TAGTACTAACGGGGTAATTGGGTTAACGTTGCTGA-3'
P7 CST (-)2	5' -AATGTGCACCTCAATCTTATCAGCAACGTTAACCCA-3'
P7 CST (+)7	5' -TAAGATTGAGGTGCACATTCACCTTCACTTTTAGTCAGATAA-3'
P7 CST (-)1	5' -GAATTTCTAATGACAACGCTATTATCTGACTAAAAGTTGAAGTG-3'
P7 CST (+)8	5' -TAGCGTTGTCATTAGAAAT'TCTGGGGAAGAATCCTGTG-3'
P7 CST (+)9	5' -CACAGGATTCTTCCCCA-3'

Oligonucleotides were ordered from Sigma. All stock solutions were 100 μ M in 1X TE. Working solutions were 10 μ M in 0.1X TE. The oligonucleotide names refer to which strand they belong to and their order from 5' to 3' of that strand. P7 CST (+)9 is actually misnamed, as it is 5' of P7 CST (-)1 on the negative strand.

2.7 Bacterial Culture

LB media was made with 10 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl. It was autoclave sterilized. LB-agar had 2% agar added. The composition of SOC media was 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2 \cdot 6H_2O$, and 20 mM glucose. Ampicillin was added to bacterial media for a concentration in the media of 100 μ g/mL. LB-Agar plates were made with a volume of approximately 25 mL in petri plates. All cultures were incubated at 37° C in autoclave sterilized containers. 5-

mL liquid cultures were in test tubes spun on a rotor during incubation. 100-mL liquid cultures were contained in 500-mL erlenmeyer flasks that were shaken at 200 RPM during incubation.

2.8 Glycerol Stocks

Bacterial glycerol stocks were made in 2 mL cryo-vials. 0.88 mL of LB media bacterial culture that had been incubated at 37° C for less than 24 hours was mixed with 0.12 mL glycerol to make a 12% glycerol solution. The solutions were flash-frozen in liquid nitrogen or frozen in dry ice, then stored at -80° C.

2.9 *Nicotiana benthamiana* Growth

N. benth seeds were planted in wet potting soil in small plastic pots with 4 squares. 3-4 seeds were placed in each square using a Pasteur pipette to transfer seeds from a water surface. Pots were covered with clear plastic wrap, and incubated in a growth chamber at 28° C. A 16 hour/8 hour day/night cycle was used. Plastic wrap was removed when the plants sprouted, and plants were watered about every other day thereafter. After growing four or more mature leaves, plants were transplanted individually to larger plastic pots and then watered until the soil was saturated to ensure transplant survival. After transplantation, watering was continued as before. Floral growths and axillary stems were pruned by hand from the plants to promote leaf growth.

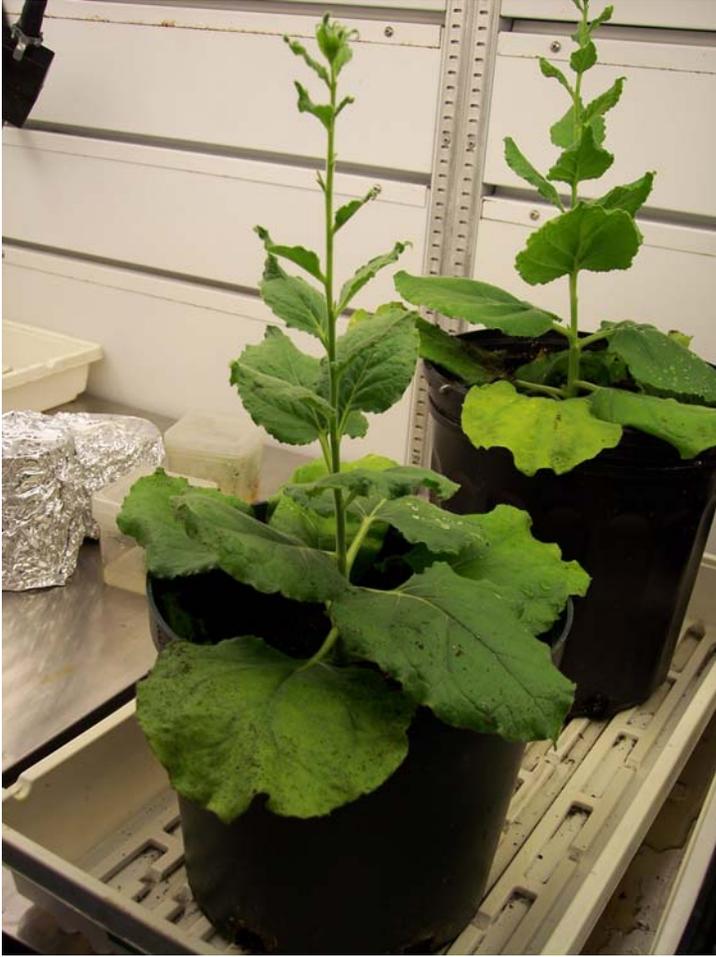


Figure 2.1: Photo of *Nicotiana benthamiana* in growth chamber.

2.10 Preparation of Chemically-Competent TOP10F' *E. coli*

Chemically-competent TOP10F' *E. coli* was prepared. 25-mL of LB media was inoculated with TOP10F' from glycerol solution at -80°C . The culture was incubated overnight at 37°C with shaking. The next day the 25-mL of culture was combined with 225-mL LB media at shook at 37°C for 1 hour 10 minutes, at which point the O.D. at 600 nm of the culture was 0.61. The culture was centrifuged for 5 minutes at 4000 RPM in a GSA Sorvall rotor, refrigerated at 4°C . The supernatant was discarded and the pellet was resuspended in 100 mL of ice-cold autoclaved 50 mM CaCl_2 . The solution was

chilled on ice for 10 min, then centrifuged as before. The supernatant was discarded and the pellet was resuspended in 20 mL of ice-cold autoclaved 20% glycerol, 40 mM CaCl₂ solution. The solution was kept on ice while 0.5 mL aliquots of the solution were made in cryo-vials and flash-frozen in liquid nitrogen. The aliquots of chemically-competent cells were stored at -80° C.

A test for transformation efficiency of the competent cells using pUC19 determined the competence of the cells to be 1.5×10^6 cfu/ μ g plasmid.

2.11 Heat-Shock Transformation of Chemically-Competent TOP10F' *E. coli*

TOP10F' competent cells prepared in 2.10 were transformed by a heat-shock method. A 0.5 mL aliquot of TOP10F' cells were thawed on ice. 50 μ L of the competent cells was combined with 2 μ L of DNA solution in a 1.5 μ L microfuge tube and kept on ice. The solution was mixed by swirling with the pipette tip. The solution was incubated on ice for 1 hour. The solution was then transferred to 37° C and incubated for 3 minutes, and then quickly put back on ice and incubated on ice for 1 minute. The solution was taken out of the ice and 1 mL of SOC media was added.

The tube was attached by tape to a spinning rotor at 37° C and incubated 1 hour. The cells were spun down by centrifuging the tube for 3 minutes at 4000 g in the 5417C centrifuge. The supernatant was removed, and the cells were resuspended in 250 μ L SOC. 20 μ L and 100 μ L of the solution was plated on separate LB-Agar-Amp plates. The plates were incubated overnight at 37° C.

2.12 Standard Plasmid DNA Miniprep Procedure

1.5 mL of a bacterial culture in LB media was micropipetted into a microfuge tube. The microfuge tube was centrifuged at 14,000 RPM in an Eppendorf (New York City, NY) 5417C centrifuge for 30 seconds. Supernatant was removed, and bacterial pellet was resuspended in 100 μ L of ice-cold GTE solution by vortexing. 200 μ L of ice-cold fresh Alkaline Lysis Solution II was added, and mixed by rapidly inverting the tube 5 times. 150 μ L of Alkaline Lysis Solution III was added and mixed by shaking by hand. The solution was stored on ice for 3-5 minutes.

The solution was centrifuged in the 5417C centrifuge for 5 minutes at 14,000 RPM. The supernatant was added by pouring or pipetting to a microfuge tube with 1 volume (450 μ L) of phenol-chloroform solution. The solution was mixed by hand-shaking, then centrifuged for 3 minutes in the 5417C at 14,000 RPM. The supernatant was transferred by pipetting to a microfuge tube with 2 volumes of 95% ethanol on ice, and stored at -80° C for a minimum of 15 minutes. The solution was then centrifuged at 4° C in an Eppendorf 5415C centrifuge at 14,000 RPM for 10 minutes. The supernatant was removed by vacuum aspiration, and the pellet was washed in 1 mL of 75% ethanol, and centrifuged as previously for 5-10 minutes. The supernatant was removed by vacuum aspiration, and the pellet was resuspended in water or 0.1X TE after evaporation of all ethanol. Plasmid solutions were stored at -20° C.

2.13 DNA Plasmid Maxiprep Procedure

Plasmid maxiprep procedure was adapted from protocol by Sosnick. A 100-mL bacterial culture was used for the maxiprep. The cell pellet was resuspended after the first

centrifugation by pipetting the solution up and down, then vortexing. The NaOH/SDS lysis solution was mixed by shaking the bottle for 5 seconds. When precipitating the RNA with LiCl in step 8, the pellet was resuspended in 3.75 mL TE buffer, then 6.25 mL of 4 M LiCl was added. The phenol-chloroform extraction mixture was not vortexed, and was instead shook by hand. It was then centrifuged at 14,000 RPM in the 5417C centrifuge for 3 minutes.

In step 15, the plasmid DNA was precipitated by adding 1 volume of isopropanol and 1 volume of 5M NH₄OAc. The precipitate was centrifuged for 15 minutes in 5415C centrifuge at 4° C at 14000 RPM. The supernatant was removed and the pellet was washed with 1 mL of 75% ethanol. The wash was centrifuged for 10 minutes as before. The supernatant was removed, and the pellet was resuspended in 0.1X TE buffer.

2.14 REDaccuTaq LA DNA Polymerase Reaction Solution

REDaccuTaq LA DNA polymerase solutions were made from 5 µL REDaccuTaq LA 10X reaction buffer, 2.5 µL of a solution containing all 4 dNTPs at 10 mM each, 2 µL of each working primer solution, 2.5 µL of Sigma REDaccuTaq LA DNA polymerase, and the template DNA solution. Sterile water was added to a total volume of 50 µL.

2.15 PfuTurbo C_X Hotstart DNA Polymerase Reaction Solution

PfuTurbo C_X Hotstart DNA polymerase solutions were made from 5 µL 10x PfuTurbo C_X Hotstart reaction buffer, 1 µL of a solution containing all four dNTPS at 10 mM each, 2 µL of each working primer solution, 1 µL Stratagene PfuTurbo C_X Hotstart

DNA polymerase, and the template DNA solution. Sterile water was added to a total volume of 50 μL .

2.16 Taq DNA Polymerase Reaction Solution

Taq DNA polymerase solutions were made from 5 μL 10X Sigma PCR reaction buffer, 0.5 μL of Sigma Taq DNA polymerase, 1 μL of a solution containing all four dNTPS at 10 mM each, 2 μL of each working primer solution, and the template DNA solution. Sterile water was added to a total volume of 50 μL .

2.17 *Arabidopsis thaliana* Growth

Arabidopsis thaliana was planted in wet potting soil by using a pipette to deposit seeds on surface. About 15-20 seeds were planted per pot. Pots were covered in clear plastic wrap and kept in growth chamber at 28° C with a 16 hour/8 hour day/night cycle. After germination of the plants, plastic wrap was opened on one edge, and the next day the plastic wrap was removed. This was done before the plants had 4 leaves. One week after germination, the plants were culled so that the plants were evenly-spaced and not crowded. Plants were watered by spray bottle with tap water, or by placing the pots in standing water in a tray and soaking the soil upwards. Watering was done when the soil surface appeared dry.



Figure 2.2: Photo of *Arabidopsis thaliana*.

2.18 Miniprep of pT1D1ΔL

Standard miniprep procedure with some changes was used for extraction of pT1D1ΔL from DH5α bacteria. The miniprep was done in duplicate. The initial centrifugation of the cells was done for 20 seconds. The solution was incubated on ice for 5 minutes after the addition of each alkaline lysis solution. No phenol-chloroform extraction was done. The DNA was precipitated by adding 800 μL of 95% ethanol. The ethanol precipitate was incubated at -80° C for 20 minutes. The solution partially froze because of inadequate mixing, and was thawed before vortexing briefly, then returned to -80° C for 10 minutes. Precipitate solution was centrifuged for 20 minutes.

The ethanol wash solution was centrifuged for 2 minutes. The pellet was resuspended in 50 μL of sterile water and 1 μL of 20 mg/mL RNase A was added. An agarose gel electrophoresis of 2 μL of plasmid solution was done to check the integrity of the plasmid, and two bands were seen, probably corresponding to supercoiled and linear.

2.19 Phosphorylation of Primers for PCR of pT1D1ΔL

Primers for PCR were phosphorylated with T4 polynucleotide kinase produced by New England Biolabs (Ipswich, MA). Each kinase solution contained 5 μL 10X T4 polynucleotide kinase buffer, 2 μL of 100 μM “NF TCV positive” or “NF TCV negative,” 0.5 μL 100 mM ATP, 1 μL 10 U/μL T4 polynucleotide kinase, and 41.5 μL sterile RNase-free water. Kinase solutions were incubated at 37° C for 30 minutes. 250 μL 95% ethanol and 10 μL 3 M sodium acetate was added to the reaction solution. The primers were precipitated overnight at -80° C. The precipitate was centrifuged in Eppendorf 5415C centrifuge for 10 minutes at 4° C at 14000 RPM. The pellet was washed in 1 mL 75% ethanol, then centrifuged as before for 5 minutes. The primers were resuspended in 50 μL sterile RNase-free water.

2.20 REDAccuTaq LA PCR of pT1D1ΔL

REDAccuTaq LA PCR was done using pT1D1ΔL with a solution as in 2.8 with one change. 2.5 μL of each phosphorylated primer solution from 2.18 was used in place of primers from working solutions. 2 μL of a 1:100 dilution of the miniprep plasmid solution of pT1D1ΔL from 2.17 was used as a template.

A heating block thermal cycler with a heated lid was used for the PCR. Initial denaturation was 95° C for 30 seconds. 40 cycles were used. Denaturation was 10 seconds at 94° C. Primers were annealed at 66° C for 20 seconds. Strand extension was done at 68° C for 12 minutes, plus 15 more seconds for each cycle after cycle 15. The

final strand extension was 20 minutes at 68° C. The PCR solutions were then kept a 4° C until removed from the thermal cyclers.

10 µL of the PCR solution was loaded on a 0.8% agarose gel. Gel electrophoresis was run at 86 V for 45 minutes. The DNA was visualized using UV-fluorescence of ethidium bromide in the gel.

2.21 Ligation of PCR Product

PCR solution from 2.20 was precipitated in ethanol after dilution to 100 µL volume. 300 µL of ice-cold 95% ethanol and 10 µL 3 M NaOAc was added to the solution in a 1.5 mL microfuge tube. Incubated precipitation solution at -80° C for 10 minutes. Centrifuged and washed with same procedure as phosphorylated primer precipitation. The DNA was resuspended in 20 µL sterile RNase-free water.

New England Biolabs T4 DNA ligase was used for blunt-end ligation of the PCR product to form pT1D1ΔLΔp8. Reaction solution was made from 10 µL of PCR product solution, 2 µL 10X T4 DNA ligase buffer (containing ATP at 100 mM), 7 µL sterile water, and 1 µL 400 U/µL T4 DNA ligase. The reaction solution was incubated overnight at 15° C.

2.22 Transformation of TOP10F' with pT1D1ΔLΔp8

2 µL of ligation solution was combined with 50 µL of chemically-competent TOP10F' prepared in 2.10. The bacterial solution was thawed on ice. Transformation procedure of 2.11 was followed. A colony from the 100-µL TOP10F' plate was

inoculated into a 5-mL LB culture with 100 µg/mL ampicillin. It was incubated overnight.

2.23 Miniprep of pT1D1ΔLΔp8

Miniprep procedure of 2.12 was followed to extract pT1D1ΔLΔp8. The ethanol precipitation was incubated at -80° C over the weekend. The DNA was resuspended in 30 µL of 0.1X TE and 1 µL 20 mg/mL RNase A. The solution was incubated for 1 hour at 37° C to degrade RNA. The plasmid solution was stored at -20° C.

2.24 P7 DNA Cassette Synthesis

Some of the oligonucleotides for the p7 DNA cassette were phosphorylated in two separate kinase reactions. 2 µL of each working solution of P7 CST (+)2 – (+)9 were combined with 2 µL 10X T4 DNA ligase buffer, 1 µL 10 U/µL T4 polynucleotide kinase, and 1 µL RNase-free water. 2 µL of each working solution of P7 CST (-)2 – (-)8 were combined with 2 µL 10X T4 DNA ligase buffer, 1 µL 10 U/µL NEB T4 polynucleotide kinase, and 3 µL RNase-free water. Both kinase reaction solutions were incubated at 37° C for 30 minutes. The kinases were then inactivated by heating at 65° C for 20 minutes.

The kinase reaction solutions were combined and 1 µL 10X T4 DNA ligase buffer, 2 µL 10 µM P7 CST (-)1, 2 µL 10 µM P7 CST (+)1, 4 µL sterile RNase-free water, and 1 µL 400 U/µL NEB T4 DNA ligase were added. The ligase reaction solution was incubated for 20 minutes at room temperature. The ligase was then inactivated by heating at 65° C for 10 minutes. The DNA was then ethanol precipitated in 300 µL 95% ethanol and 10 µL 3 M NaOAc. The solution was stored at -80° C for 5 days. The

precipitate was then centrifuged in 5415C centrifuge at 4° C for 10 minutes at 14,000 RPM. The pellet was washed with 1 mL 75% ethanol, and was centrifuged as previously. The pellet was resuspended in 30 µL sterile RNase-free water. The maximum yield of the cassette synthesis is 3.6 µg. The maximum concentration of DNA in solution is 120 ng/µL.

2.25 PfuTurbo C_X Hotstart PCR of p7 Cassette and pT1D1ΔLΔp8

The cassette from 2.24 was used in PCR with PfuTurbo C_X Hotstart using a solution described in 2.15. “NF p7 USER L” and “NF p7 USER R” were the primers used in the PCR. 1 µL of a 1:10 dilution of the cassette solution was used for the template DNA. No-template and no-polymerase controls were also prepared.

For the PCR protocol, the initial denaturation was 2 minutes at 95° C. 30 cycles were done; the denaturation was 95° C for 30 seconds; annealing was 55° C for 30 seconds; extension was 72° C for 1 minute. The final extension was 72° C for 10 minutes. The solution’s temperature was maintained at 4° C until the PCR was removed.

10 µL of each PCR was combined with 2 µL of 6x loading buffer and loaded on to a 1.2% agarose gel with ethidium bromide in TAE buffer. Gel electrophoresis was run at 80 V for 45 minutes. The DNA was visualized by UV-fluorescence.

pT1D1ΔLΔp8 was used as the template for PfuTurbo C_X Hotstart PCR. The solution was made as described in 2.15. The primers were “TCV USER pos” and “TCV USER neg.” 2 µL of a 1:100 dilution of the pT1D1ΔLΔp8 was used for the DNA template. A no-template and no-polymerase control were also made. PCR protocol was as for the PCR of the cassette, except that the extension time was 9 minutes. 10 µL of each

PCR with 2 μL 6X loading buffer was loaded on a 0.8% agarose TAE gel, and electrophoresis was run at 95 V for 30 minutes.

2.26 USER Enzyme Reaction

New England Biolabs USER enzyme was used to excise the deoxy-uracil base and 5' nucleotides in the PCR products from 2.25. 0.6 μL of the p7 cassette PCR, 2.75 μL of the pT1D1 Δ L Δ p8 PCR, 0.77 μL 10X PfuTurbo C_X Hotstart reaction buffer, 6.88 μL sterile water, and 1 μL 1 U/ μL USER enzyme were combined. The solution was incubated at 37° C for 15 minutes, then incubated at room temperature for 15 minutes. 1 μL of 10X T4 DNA Ligase buffer with 10 mM ATP and 1 μL of 400 U/ μL T4 DNA ligase from NEB was added to the USER reaction solution and the solution was incubated at room temperature for 30 minutes. The solution was stored at -20° C. The intended product of the USER reaction was named pTCVNF1.

2.27 Transformation of TOP10F' With USER Enzyme Reaction Solution

TOP10F' bacteria were transformed with the USER enzyme reaction solution using the procedure described in 2.11. 100 μL of TOP10F' cells thawed on ice were combined with 11 μL of the USER enzyme reaction solution from 2.26.

2.28 Construction of pTCp7 Δ p8

Modifications were made to pT1D1 Δ L to create pTCp7 Δ p8. pTCp7 Δ p8 is similar to the plasmid created in 2.27, except it does not incorporate alterations to the sequence of p7 because the p7 insert was created from RT-PCR. A REDAccuTaq LA PCR solution

was made as in 2.20, except 2 μL of each working primer solutions instead of phosphorylated primers were used, and 2 μL of a 1:100 dilution of the plasmid miniprep solution of pT1D1 ΔL from 2.18 was used for the DNA template. The same thermal cycler settings were used, except only 30 cycles were done. Agarose gel electrophoresis showed the presence of the 8 kb PCR product.

The PCR product was phenol-chloroform extracted, then precipitated in 95% ethanol and NaOAc, and washed in 100% ethanol. The pellet was resuspended in 5 μL 10X T4 DNA Ligase buffer, with 10 mM ATP, 44.5 μL sterile water, and 0.5 μL 10 U/ μL T4 polynucleotide kinase from NEB. The reaction solution was incubated at 37° C for 1 hour. The solution was heated at 68° C for 10 minutes to inactivate the kinase. The DNA was then phenol-chloroform extracted and precipitated in ethanol as before. The DNA pellet was resuspended in 90 μL TE buffer. 45 μL of the DNA solution, 10 μL of T4 DNA Ligase buffer, 0.67 μL 400 U/ μL T4 DNA ligase (NEB), and 44.3 μL of sterile water were combined and incubated overnight in at 4° C. The DNA was again phenol-chloroform extracted and precipitated in ethanol. The DNA was resuspended in 8 μL sterile water. 6 μL of the solution was used to transform 50 μL of TOP10F' cells as described in 2.11. 5-mL of LB media was inoculated and incubated overnight at 37° C on a spinning rotor. A plasmid miniprep was done as described in 2.12. The pellet was resuspended in 30 μL 0.1X TE. The plasmid in this solution is a different clone of pT1D1 $\Delta\text{L}\Delta\text{p}8$ than the one used to make pTCVNF1.

CarMV virions were purified from *N. benthamiana* leaves using the rapid mini-sample procedure described in Cartwright (2006), except that the polyethylene glycol (PEG) precipitate was resuspended in 1 mL 0.05 M NaOAc, pH 5.2, and stored overnight at 4°

C. The solution was then centrifuged at 14000 RPM for 10 minutes in Eppendorf 5417C. The supernatant was poured into a 1.5 mL microfuge tube containing 0.3 mL 40 % PEG 8000, 1 M NaCl. This solution was vortexed and incubated on ice for 10 minutes. The solution was centrifuged again as previously, and the pellet was resuspended in 0.1 ml 0.01 M NaOAc, pH 5.5.

64 μ L of this virion solution was combined with 100 μ L 10X STE solution (10X: 0.5 mM Tris, 0.01 M NaEDTA, 1.0 M NaCl, pH 7.4), 50 μ L 10% SDS, and 286 μ L sterile water. The solution was heat shocked for 30 seconds at 65° C. 1.0 mL of phenol-chloroform was added, and solution was vortexed 30 second and shaken by hand for 1 minute. The solution was the centrifuged in 5417C for 3 minutes at 14000 RPM. The aqueous phase was transferred to a microfuge tube containing 1 mL phenol-chloroform and shaken by hand for 1 minute and centrifuged as before. The aqueous phase was transferred to a microfuge tube containing 50 μ L 3 M NaOAc and 1 mL ice-cold 95% ethanol. The solution was stored at -20° C for two days. Solution was centrifuged in 5415C at 4° C for 10 minutes at 14000 RPM. Supernatant was removed, and RNA was resuspended in 25 μ L RNase-free water. 10 μ L of RNA solution was used for reverse transcription (RT) with random hexamer oligonucleotide primers using Qiagen (Valencia, CA) Omniscript Reverse Transcriptase and the protocol in the product manual. The RNase-inhibitor used in the reverse transcription was produced by Promega (Madison, WI).

2 μ L of the RT reaction was used in a PfuTurbo C_X Hotstart PCR otherwise identical to the p7 cassette PCR in 2.25. 1 μ L of a 1:10 dilution of the pT1D1 Δ L Δ p8 plasmid miniprep in 2.28 was used in a PfuTurbo C_X Hotstart PCR as for the plasmid in

2.25, except 35 cycles were used, and the extension time and final extension times were 12 minutes.

The PfuTurbo C_X Hotstart PCR products were used in a USER enzyme reaction. 2 μL of each PCR, 8 μL of 1X PfuTurbo C_X Hotstart reaction buffer, and 1 μL of 1 U/μL USER enzyme were combined, and the solution was incubated 15 minutes at 37° C followed by 15 minutes at room temperature. The USER reaction solution was used for heat-shock transformation of TOP10F' as described in 2.11. 5 mL of LB media was inoculated with a colony of transformed TOP10F'. The culture was incubated on a spinning rotor overnight at 37° C. A plasmid miniprep was then done as described in 2.12. The plasmid was named pTCp7Δp8. The presence of an insert was verified by a BglII and SpeI double restriction digest.

2.29 PCR Screening of pTCVNF1 Transformed TOP10F'

12 Taq PCR reaction solutions were prepared as described in 2.16. 2 μL of a 1:100 dilution of the miniprep of pTCVp7Δp8 in 2.28 was used as a positive control. A no-template PCR was a negative control. The 10 other PCR solutions were filled to volume, and micropipette tip were scraped against separate colonies from the plates cultured in 2.26, then swirled in the PCR solution and ejected into a test tube containing 5 mL of LB media. The thermal cycler protocol was as the cassette PCR in 2.25, except cycle denaturation temperature was 94° C and the final extension was 5 minutes. A 1.2% agarose gel in TAE buffer was used for gel electrophoresis. 10 μL of each PCR with 2 μL of 6X loading buffer containing ethidium bromide was loaded into the wells. Electrophoresis was run at 95 V for 30 minutes, and the DNA was visualized with UV-

fluorescence. The tubes each inoculated with one of 3 colonies that were positive on the PCR screens and one colony that gave an anomalous PCR product were incubated overnight. Plasmid minipreps were done on the cultures as described in 2.12, with two phenol-chloroform extractions. The plasmids were resuspended in 39 μL 0.1X TE buffer and μL of 20 mg/mL RNase A. The solutions were incubated at 37° C for 20 minutes to degrade RNA.

Taq PCR screening was repeated on the plasmids from the minipreps. The PCR controls were also repeated. 2 μL of a 1:100 dilution of the plasmid miniprep was used as the template. Gel electrophoresis was done as previously, except the gel contained ethidium bromide instead of the loading buffer.

2.30 Restriction Digest of Candidate pTCVNF1

BglII and SpeI were used in a double digest of a plasmid which was positive for the USER reaction insert in the second PCR screen of 2.29. SpeI was also used to determine the plasmid length. The restriction enzymes were purchased from New England Biolabs. A double restriction digest with BglII and SpeI were also done of pT1D1 ΔL and pT1D1 $\Delta\text{L}\Delta\text{p}8$. A no-digest control of the candidate plasmid was prepared by combining 5 μL of the plasmid with 4 μL 10X NEB buffer 2, 0.4 μL 100X bovine serum albumin (BSA), and 30.6 μL sterile water. It was incubated 1 hour at 37° C.

The double restriction digest solution of the pTCVNF1 candidate plasmid contained 1 μL 10 U/ μL SpeI, 3 μL 10 U/ μL , 2.5 μL plasmid miniprep solution, 5 μL 10X NEB buffer 2, 0.5 μL 100X BSA, and 38 μL sterile water. The reaction solution was incubated at 37° C for two hours.

The SpeI digest of the pTCVNF1 candidate plasmid contained 1 μL 10 U/ μL SpeI, 5 μL of the plasmid miniprep, 4 μL 10X NEB buffer 2, 0.4 μL 100X BSA, and 29.6 μL sterile water. It was incubated for 1 hour at 37° C.

The double-digests of pT1D1 ΔL and pT1D1 $\Delta\text{L}\Delta\text{p}8$ each contained 1 μL 10 U/ μL BglII, 1 μL 10 U/ μL SpeI, 5 μL of the plasmid miniprep, 4 μL 10X NEB buffer 2, 0.4 μL 100X BSA, and 28.6 μL sterile water. The reaction solution was incubated for 4 hours at 37° C.

A 0.8% agarose gel in TAE buffer was used for gel electrophoresis. 6 μL of the SpeI digest of the candidate plasmid and 1.2 μL of 6X loading buffer were combined and loaded on to the gel. The same amount of the control digest was loaded. 15 μL of the double-digest of the candidate plasmid with 3 μL 6X loading buffer was loaded on to the gel. 5 μL of the double digest of pT1D1 $\Delta\text{L}\Delta\text{p}8$ and 1 μL 6X loading buffer was combined and loaded on to the gel. 10 μL of the double-digest of pT1D1 ΔL was combined with 2 μL 6X loading buffer and loaded on to the gel. Gel electrophoresis was run at 75 V for 1 hour.

2.31 Electroporation of DH5 α With pT1D1 ΔL

DH5 α electro-competent cells were transformed with pT1D1 ΔL using electroporation. The electroporation apparatus used was Cell-Porator electroporation apparatus from GIBCO BRL Life Technologies (corp. defunct) with a Cell-Porator voltage booster, also from Life Technologies. The gap width on the bosses was 0.1 cm. A 1:25 mixture of pT1D1 ΔL miniprep from 2.18 and DH5 α electro-competent cells thawed

on ice was made, and approximately 20 μL was pipetted onto the bosses of an electroporation cuvette chilled on ice in the electroporation apparatus. The capacitance used was 330 μF , high resistance was used, and the voltage booster was set at 4 Ω . The charge was triggered at 430 V. The applied voltage was 1.94 kV.

The electroporated cells were mixed with 1 mL SOC media and incubated on a rotor at 37° C for 2 hours. 20 μL and 100 μL of the solution was plated on to separate LB-agar-ampicillin plates. Plates were incubated overnight.

2.32 Plasmid Maxiprep of pT1D1 Δ L

5-mL of LB media with ampicillin was inoculated with a colony from the plate of electroporated DH5 α from 2.31, and incubated overnight. A glycerol stock was made from the liquid culture. The glycerol stock was used to inoculate a 100-mL culture of LB media with ampicillin. The 100-mL culture was incubated overnight. A plasmid maxiprep was then done on the culture as described in 2.13. The DNA was resuspended in 200 μL of 0.1X TE buffer. UV-spectrophotometry estimated the concentration of DNA to be 3.62 $\mu\text{g}/\mu\text{L}$.

2.33 Plasmid Maxiprep of pTCVNF1

A 100-mL culture of LB media with ampicillin was inoculated with a colony from a plate streaked with TOP10F' transformed with pTCVNF1, from 2.29. The 100-mL culture was incubated overnight. The culture was used for a plasmid maxiprep using the procedure in 2.13. The plasmid was resuspended in 100 μL 0.1X TE buffer. UV-spectrophotometry determined the concentration to be 3.79 $\mu\text{g}/\mu\text{L}$.

2.34 Glycerol Stocks

Pairs of glycerol stocks were made of DH5 α transformed with pT1D1 Δ L, TOP10F' transformed with pT1D1 Δ L Δ p8, TOP10F' transformed with pTCp7 Δ p8, and TOP10F' transformed with pTCVNF1. All glycerol stocks were stored at -80° C.

2.35 XbaI Restriction Digest of pT1D1 Δ L and pTCVNF1

XbaI restriction digests of pT1D1 Δ L and pTCVNF1 were done to linearize the plasmid and fix the end of the T7 RNA polymerase transcribed region. The XbaI restriction enzyme was from NEB. The reaction solutions both contained 4 μ L 20 U/ μ L XbaI, 10 μ L 10X NEB buffer 2, and 1 μ L 100X BSA. 3.87 μ L of pT1D1 Δ L and 3.69 μ L of pTCVNF1 from the maxipreps in 2.32 and 2.33 were used in their respective digests, approximately 14 μ g of each plasmid. Sterile RNase-free water was added to each reaction solution for a 100 μ L total volume. The reaction solutions were incubated at 37° C for 1 hour.

Each reaction solution was diluted with 100 μ L of sterile RNase-free water to a total volume of 200 μ L, then a phenol-chloroform extraction was done with 200 μ L of phenol-chloroform. The solutions were shaken by hand, and centrifuged in the 5417C at 14000 RPM for 3 minutes. The aqueous phases were transferred to separate microfuge tubes, and 500 μ L of 95% ethanol and 20 μ L 3M NaOAc was added to precipitate the DNA. The precipitation solutions were incubated at -80° C overnight. The solutions were then centrifuged at 4° C in 5415C at 14000 RPM for 10 minutes, then the supernatant was removed. The pellet was washed in 1 mL 75% ethanol and centrifuged as before.

The pellet of cut pT1D1ΔL DNA was resuspended in 20 μL 0.1X TE buffer, and the cut pTCVNF1 DNA was resuspended in 25 μL 0.1X TE buffer.

2.36 T7 RNA Polymerase Viral RNA Transcription

T7 RNA polymerase was used to transcribe viral RNA from the plasmids cut with XbaI in 2.35. The T7 RNA polymerase was produced by Promega. Both solutions contained 20 μL 5X transcription optimized buffer from Promega, 5 μL 80 U/μL T7 RNA polymerase, 2 μL 40 U/μL RNase-inhibitor from Promega, and 10 μL 10 mM of all 4 NTPs. 20 μL of XbaI-cut pT1D1ΔL from 2.35, approximately 14 μg, was used in one reaction. The other reaction used 25 μL of XbaI-cut pTCVNF1 from 2.35, also approximately 14 μg. Sterile RNase-free water was added for a total reaction volume of 100 μL.

The T7 RNA polymerase reactions were incubated at 37° C for 2 hours. The reaction solutions were phenol-chloroform extracted in one volume of phenol-chloroform and centrifuged, then the aqueous phase was transferred and 0.1 volumes of 3M NaOAc was added. 3 volumes of 95% ethanol was added, and the nucleic acids were precipitated at -80° C overnight. The precipitate was centrifuged and washed as in 2.35. The precipitated DNA and RNA was resuspended in sterile RNase-free water. The RNA transcribed from pTCVNF1 was resuspended in 100 μL, and the RNA transcribed from pT1D1ΔL was resuspended in 50 μL. The difference in volume was based on the observed difference in pellet size.

The absorbance of the solutions at 260 nm and 280 nm was measured by 1:250 dilution in sterile RNase-free water. 1 μL of a 1:10 dilution of each solution was mixed

with 4 μL sterile RNase-free water and 1 μL 6X loading buffer was loaded on to a 0.8% agarose gel in TAE buffer, to check that the RNA was present as a single band and was not fragmented. 3 μL of a 1:10 dilution of each solution with 2 μL sterile RNase-free water and 1 μL 6X loading buffer was also loaded on to the gel. Electrophoresis was run at 105 V for 15 minutes.

2.36 Inoculation of Viral RNA

Viral RNA was prepared for inoculation by mixing the RNA solutions from 2.36 in equal volumes with 2X inoculation buffer on Parafilm. Mock inoculations were done by using sterile RNase-free water in place of the RNA solution. 2X inoculation buffer was composed of 0.1 M glycine, 0.06 M K_2HPO_4 , and 2% celite. The celite is abrasive to the plant surface, and creates holes for entry of pathogenic particles into the cells by breaking the hair-like structures on the leaf surface. The inoculation buffer was vortexed thoroughly before mixing with RNA solution, to suspend the insoluble celite.

Large leaves midway between the crown and base of mature *N. benthamiana* were inoculated. Two plants were used. One plant had three leaves inoculated with wild-type TCV RNA and one leaf was mock-inoculated. The other plant had three leaves inoculated with mutant TCV RNA and one leaf mock-inoculated. Inoculation was done by touching the RNA-inoculation buffer solution with a finger in a latex glove, then gently rubbing the solution on to the top surface of a leaf by rubbing the leaf between the finger and thumb.

A. thaliana was inoculated at least 18 days after germination. The 3 oldest true leaves were inoculated by gently rubbing the top of the leaf with a glass stirring rod that

had been touched to a mixture of inoculation buffer and RNA solution. The leaf was backed against the end of a metal spatula or a thin piece of cardboard.

3. Results

3.1 Strategy for Genetic Modification

The goal of this project was to create a mutant *Turnip crinkle virus* in which the gene for the movement protein p8 was replaced by the *Carnation mottle virus* gene for the movement protein p7, for the purpose of determining if p8 was required for *Turnip crinkle virus* to suppress the hypersensitive response. In order to accurately test this hypothesis, the other genes of *Turnip crinkle virus* had to have no significant changes, including changes in expression level.

Three factors were accounted for in the inactivation of p8 and insertion of p7: 1) the genetic modification must not change the amino acid sequence of either of the overlapping genes (p9 and p88); 2) The translation of p9 must be preserved at its normal level; 3) p7 must be inserted in a way that ensures translation of the gene is similar to the translation of p8 in the wild-type.

(1) and (3) were accomplished by inserting the p7 gene into the region of the p8 gene that does not overlap other genes, and inactivating p8 by altering the start codon of p8 in a manner that used codon redundancy to avoid altering the amino acid sequence of p88. The easiest way to eliminate the start codon of p8 was by single-nucleotide substitution using PCR. The primers NF TCV Positive and NF TCV Negative amplified the entire clone containing the full length cDNA, pT1D1ΔL, with a base-pair mismatch in the sequence of NF TCV Positive, changing the adenosine base in the start codon of p8 to a guanine. The ligated product of this reaction was named pT1D1ΔLΔp8.

PCR Single-Nucleotide Substitution

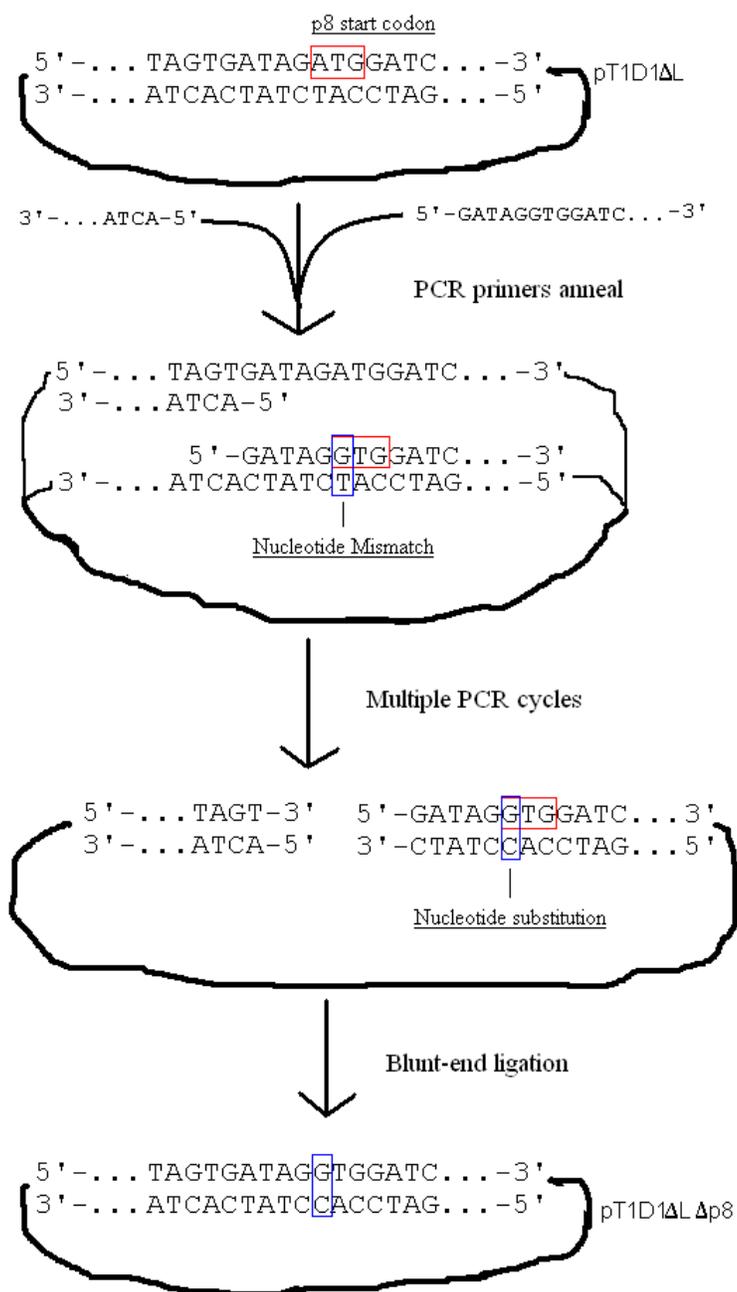


Figure 3.1: PCR single-nucleotide substitution. The first base of the start codon of p8 was changed from adenosine to guanine in the plasmid PT1D1ΔL, eliminating the start codon and p8 ORF. This PCR created pT1D1ΔLΔp8.

To create an insert of CarMV p7 that would not interfere with translation of TCV p9 by leaky scanning, a DNA cassette matching the sequence of p7 and the nearby regions of the CarMV genome was created from oligonucleotides. The oligonucleotides were designed so that the cassette sequence would differ from the sequence in CarMV by the removal of two potential translational start sites following the p9 start site, and the removal of the end of the 1.45 kb sgRNA promoter. The start sites, one of which was for the CarMV p9 gene, were removed to protect leaky scanning translation of TCV p9. As in the first step, this was done by single-nucleotide substitutions that used codon redundancy to avoid changing the amino acid sequence of p7. The p7 gene contained the entire 1.45 kb sgRNA promoter (Wang & Simon, 1997). The promoter had to be disabled to prevent a third sgRNA from being made, which would have had an effect on p9 expression that would confound the research to be done with the mutant virus. The 3' end of the promoter was outside the coding sequence of p7, and 24 bases after p7 were not included in the cassette. The sequence the p7 USER R primer binds to is the sequence that follows these 24 bases, so the exclusion of the 24 bases is equivalent to a deletion mutation.

p7-Insert DNA Cassette

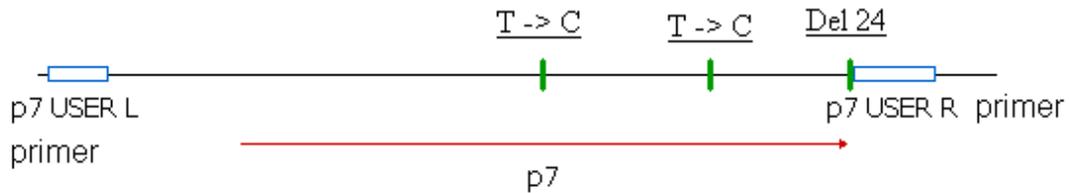


Figure 3.2: Map of p7-insert cassette. Changes from original sequence are marked by vertical green lines. Blue squares represent binding sites for PCR primers. Figure created with Clone Manager 7.

An insert was created from the cassette using the dU-containing primer pairs in PCR on the cassette and pT1D1ΔLΔp8. Treating the PCR products with USER enzyme created unique sticky ends, allowing the orientation-specific insertion of the p7 gene into pT1D1ΔLΔp8. The USER enzyme removes the dU nucleotide, causing the 9 bases on the 5' end of the PCR products to dissociate from the DNA, exposing complementary sequences on the insert and plasmid. The nicks between the insert and plasmid were repaired by T4 DNA ligase. Because the start codon of p8 was eliminated, the start codon of p7 is the first start codon in an initiation-context sequence on the 1.7 kb sgRNA and should be translated at levels similar to p8 in wild-type TCV. The plasmid created by this recombination was named pTCVNF1. This plasmid was used to transcribe viral RNA for infection of plants with the mutant Turnip Crinkle Virus.

Sequence Insertion with USER Enzyme

5'-ATTAGATCU...ATTAGAAAT-3'
3'-TAATCTAGA...UAATCTTTA-5'

Cassette PCR product

5'-...ATTAGATCT-3' 5'-ATTAGAAAU...-3'
3'-...UAATCTAGA-5' 3'-TAATCTTTA...-5'

pT1D1ΔLΔp8 PCR product

↓ USER Enzyme Reaction

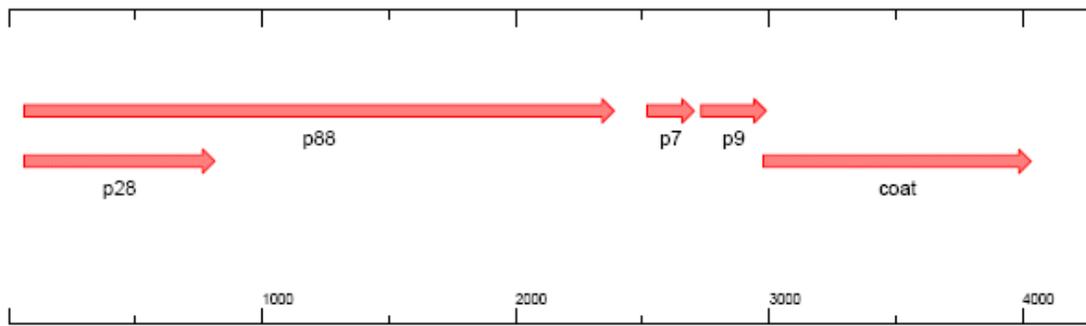
5'-...ATTAGAAAT-3'
3'-TAATCTAGA...-5'

5'-...ATTAGATCT-3' 5'-...-3'
3'-...-5' 3'-TAATCTTTA...-5'

↓ Annealing of
Sticky-Ends

5'-...ATTAGATCT]...ATTAGAAAT]...-3'
3'-...[TAATCTAGA...[TAATCTTTA...-3'

Figure 3.3: Recombination of the PCR products of pT1D1ΔLΔp8 and p7-insert cassette with USER enzyme reaction.



TCV: p8-, CarMVp7+ (4286 bps)

Figure 3.4: Gene map of mutant TCV transcribed from pTCVNF1.

3.2 Elimination of the p8 Start Codon With PCR Single-Nucleotide Substitution

REDAccuTaq LA DNA polymerase from Invitrogen was used for the PCR to disrupt the p8 start codon. The length of the expected PCR product was ~8 kb. The results of the PCR are shown in figure 3.5.

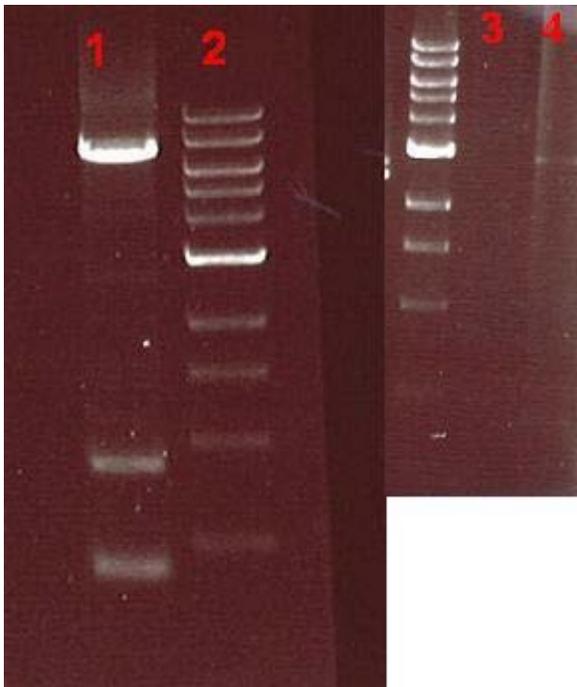


Figure 3.5: Gel electrophoresis of PCR single-nucleotide substitution. Lane 1 is the PCR. Lane 2 is a 1-kb ladder. The top 6 bands on the ladder are 10-kb, 8-kb,

6-kb, 5-kb, 4-kb, and 3-kb. The mass of the 3-kb band is 125 ng of DNA. The mass of DNA in the PCR product band at 8-kb is estimated to be >250 ng. Lane 3 is a no-template control from a prior REDAccuTaq PCR that used the same primers and the same thermal cycler settings, except that 30 cycles were used instead of 40 cycles. Lane 4 is a failed PCR that was intended to make pT1D1ΔLΔp8. The control in lane 3 was the control for this PCR. This gel electrophoresis was a 0.8% agarose gel run at 80 V for 90 minutes.

Gel electrophoresis showed a few non-target PCR products were present in the PCR, but they can be disregarded due to their small size and low ratio to the intended PCR product. The concentration of DNA in the PCR was estimated to be 25 ng/μL, with the total yield of the PCR being 1.25 μg.

After cloning of the plasmid in TOP10F' bacteria, the plasmid was extracted from the bacteria by a plasmid miniprep, and some of the extracted DNA was digested with the restriction enzyme SpeI, a single-cutter for pT1D1ΔLΔp8. Gel electrophoresis of the cut plasmid showed that the plasmid was the expected length, ~8 kb.

3.3 PCR Amplification of the p7 Cassette with dU-Containing Primers

PfuTurbo C_X DNA Polymerase was used for PCR of the p7 cassette to create the insert for the USER reaction. The PCR product should be 272 bp in size. Figure 3.6 shows that the intended cassette PCR product was the only product. Other gel electrophoresis showed the concentration of product to be 42 ng/μL.

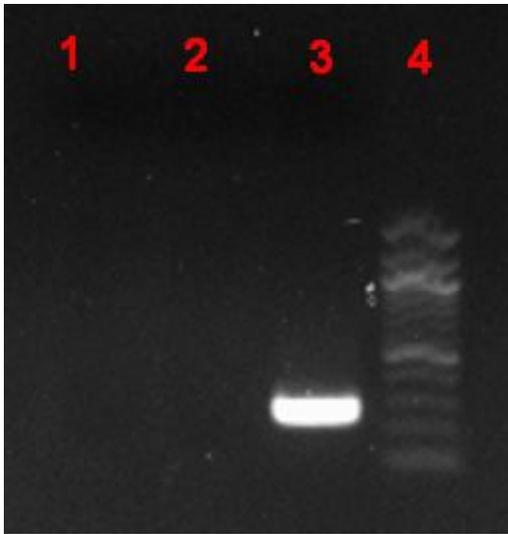


Figure 3.6: Gel electrophoresis of p7 cassette PCR. Lane 1 is a no-polymerase control; lane 2 is a no-template control; lane 3 is the PCR of the cassette; and lane 4 is a 100-bp ladder. The bottom four bands on the ladder are 100 bp, 200 bp, 300 bp, and 400 bp.

3.4 PCR of pT1D1 Δ L Δ p8 With dU-Containing Primers

PfuTurbo C_X DNA Polymerase was used for PCR of pT1D1 Δ L Δ p8 to create the “vector” for the USER reaction. In figure 3.6, gel electrophoresis showed the expected 8-kb PCR product was the major product of the PCR. A later gel electrophoresis allowed estimation of its concentration to be 25 ng/ μ L. Some PCR products of length greater than or less than the expected PCR product were also present. The no-template control PCR had a 5 kb PCR product. This product does not match the size of the PCR product of any PCR done. The contaminating PCR product is present in much lower concentration than the PCR product of pT1D1 Δ L Δ p8, and it is much smaller, so recombination with the contaminating product is not as likely, and clones of recombinant with the contaminant should be screened out by plasmid size determination.

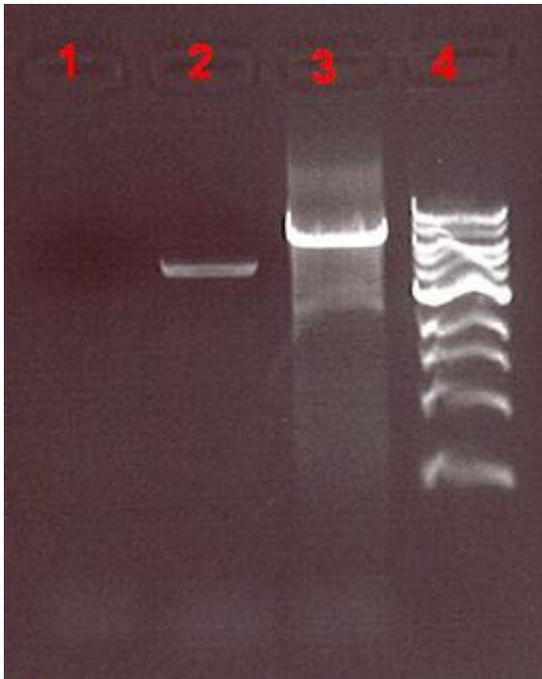


Figure 3.7: Gel electrophoresis of pT1D1 Δ L Δ p8 PCR. Lane 1 contains a no-polymerase control PCR. Lane 2 contains a no-template control PCR. Lane 3 contains the PCR. Lane 4 contains a 1-kb ladder.

3.5 Screening of Recombinant Plasmid Clones

Plated colonies of TOP10F' transformed with the USER reaction were screened by Taq PCR using the p7 insert primers. Figure 3.7 shows the electrophoresis of the PCR products.

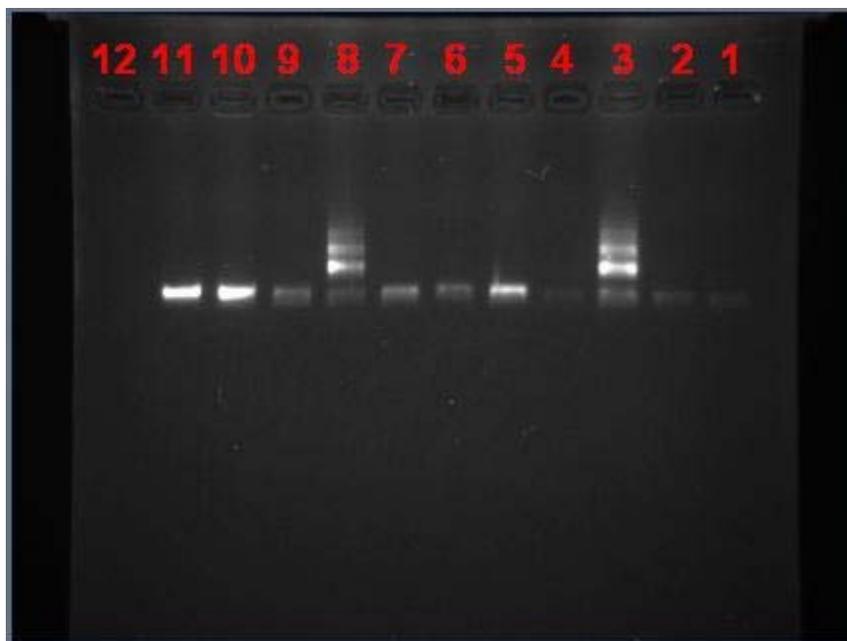


Figure 3.8: Gel electrophoresis of Taq PCR screening of colonies. Lane 12 is a no-template control. Lane 11 uses pTCp7 Δ p8 as a template. Lanes 1 - 10 correspond to pFinal5.1 – pFinal5.10.

All colonies screened were positive for the insert except 3 and 8, which had multiple PCR products. Colony 1, 5, 8, and 10 were cultured in 5-mL LB media. The plasmids were extracted, and the same Taq PCR was done with the extracted plasmid as a template.

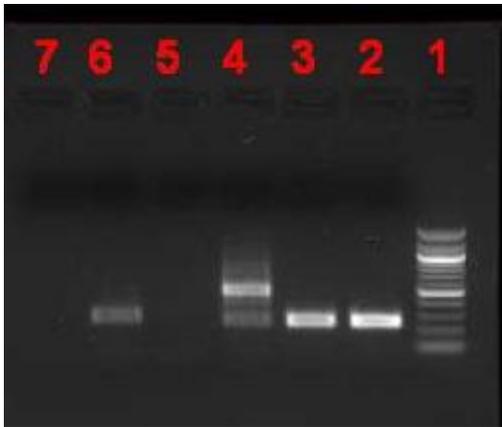


Figure 3.9: Gel electrophoresis of diagnostic Taq PCR of insert. Lane 1 is a 100-bp ladder. The bottom 4 bands are 100 bp, 200 bp, 300 bp, and 400 bp. Lane 2 is the PCR of pFinal5.1. Lane 3 is the PCR of pFinal5.5. Lane 4 is the PCR of pFinal5.8. Lane 5 is the PCR of pFinal 5.10. Lane 6 is the PCR of pTCp7 Δ p8. Lane 7 is a no-template control PCR.

The PCR results in figure 3.8 showed that pFinal5.1 and pFinal5.5 contained the insert. The PCR of pFinal5.8 again gave multiple PCR products. pFinal5.1 was selected for further tests.

SpeI is a single-cutter of all plasmids used, with a restriction site in the TCV genome downstream of the inserted DNA. BglII does not cut pT1D1 Δ L or pT1D1 Δ L Δ p8, but the insertion of the p7 cassette PCR product introduces a single BglII restriction site. An SpeI restriction digest was done of pFinal5.1 to determine its size, and a double-digest with SpeI and BglII was done to verify the location of the insert. The double-digest was also done on pT1D1 Δ L, pT1D1 Δ L Δ p8, and pTCp7 Δ p8. A 1.7 kb fragment was expected for the double digest if the insert is in the correct position and orientation. The plasmids total size should be ~8 kb. A gel electrophoresis of the digest products is shown in figure 3.9.

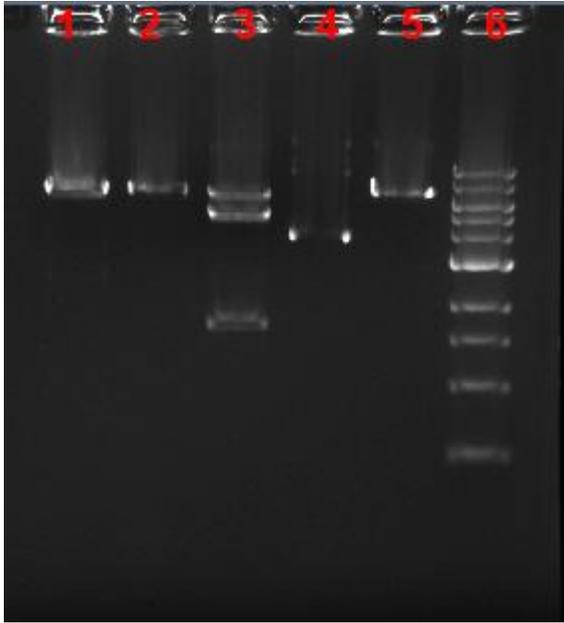


Figure 3.10: Gel electrophoresis of restriction digests of plasmids. A 0.8% agarose gel in TAE buffer was run at 75 V for 1 hour. Lane 1 is a BglIII-SpeI digest of pT1D1ΔL. Lane 2 is a BglIII-SpeI digest of pT1D1ΔLΔp8. Lane 3 is a BglIII-SpeI digest of pTCVNF1. Lane 4 is a control digest of pTCVNF1. Lane 5 is a SpeI digest of pTCVNF1. Lane 6 is a 1-kb ladder. The bottom 4 bands of the ladder are 0.5 kb, 1 kb, 1.5 kb, and 2 kb.

The BglIII used was consistently giving partial digests, and was likely to have degraded, being assayed last in 1996. As demonstrated by the SpeI-only digest of pTCVNF1, SpeI and BglIII each cut the plasmid once, while BglIII does not cut pT1D1ΔL or pT1D1ΔLΔp8. The fragment size corresponds to that expected for the presence of the insert. Final5.1 was selected for expression of TCV mutant viral RNA, and was renamed pTCVNF1.

3.6 Viral RNA Transcription

The 3' end of the dsDNA copy of Turnip Crinkle Virus on pT1D1ΔL and pTCVNF1 was cut with XbaI to limit the template for RNA transcription to the virus'

sequence. Gel electrophoresis was done of a sample of the digest to verify that XbaI had fully linearized the plasmid.

The RNA concentration after transcription was measured by UV spectrophotometry. The pTCVNF1-transcribed RNA O.D. at 260 nm was 0.3417 for a 1:250 dilution, and the $A_{260}:A_{280}$ ratio was 1.87, indicating a high purity. The pT1D1 Δ L-transcribed RNA O.D. at 260 nm was 0.1249 for a 1:250 dilution and the $A_{260}:A_{280}$ ratio was 1.92. With an extinction coefficient of 40 $\mu\text{g}/\text{mL}$ for ssRNA, the concentration of pTCVNF1-transcript was 3.417 $\mu\text{g}/\mu\text{l}$ and the concentration of pT1D1 Δ L-transcript was 1.249 $\mu\text{g}/\mu\text{L}$. However, the spectrophotometry overestimates the concentration of RNA because of the presence of the original plasmid DNA in the solution. The solution of pTCVNF1-transcript should contain 0.12 - 0.14 $\mu\text{g}/\mu\text{L}$ DNA, and pT1D1 Δ L-transcript solution should contain 0.24 – 0.28 $\mu\text{g}/\mu\text{L}$ DNA.

RNA was run in an agarose gel electrophoresis to verify that the transcript was intact. Imaging of the gel with ethidium bromide UV-fluorescence in figure 3.10 showed that distinct bands were present at the same size in both RNA solutions. The additional 2 bands in the pTCVNF1-transcript solution are likely the template plasmid DNA in linearized and circular or supercoiled form.

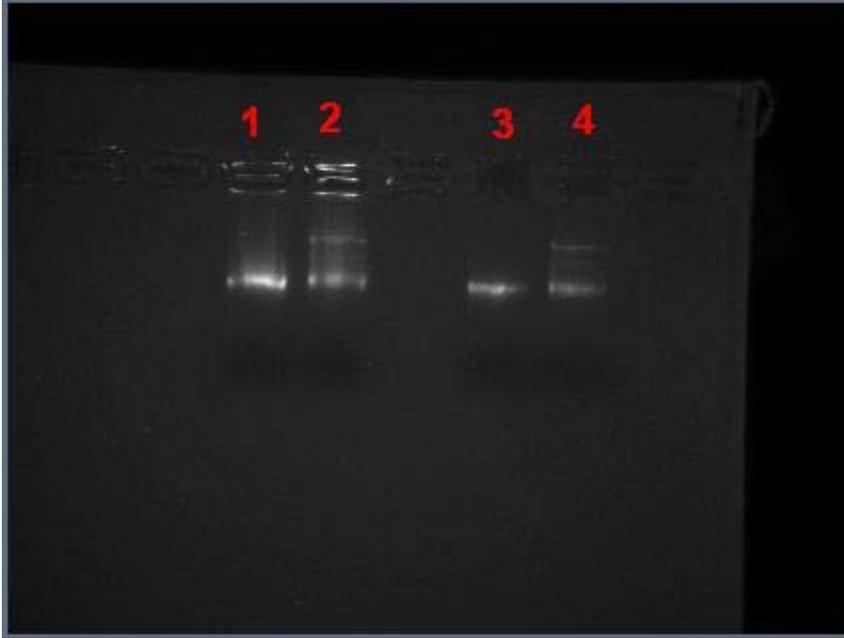


Figure 3.11: Agarose gel electrophoresis of viral RNA transcripts. Lane 1 and lane 2 are 3 μL of TCV wild-type RNA and mutant RNA, respectively, combined with 2 μL sterile RNase-free water and 1 μL 6x loading buffer. Lane 3 and lane 4 are 1 μL of TCV wild-type RNA and mutant RNA, respectively, combined with 4 μL sterile RNase-free water and 1 μL 6x loading buffer.

4. Discussion

4.1 Difficulties in Creating the Mutant *Turnip crinkle virus* Genome

Several technical problems were encountered in the process of creating the mutant TCV. One problem was that transformation with the USER enzyme reaction repeatedly failed to produce a clone of pTCVNF1. The original plan to create the mutant TCV involved two further single-nucleotide substitution PCRs with pTCp7 Δ p8, but multiple PCR attempts failed to produce the intended product, and the cassette was used instead.

The USER enzyme reaction successfully produced a clone of pTCp7 Δ p8 the first time it was attempted, but later attempts to create pTCVNF1 were initially not successful. Some possible causes for the difficulties may have been degradation of the primers used in the PCR, non-target products of the PCR of pT1D1 Δ L Δ p8 interfering with the reaction, or the use of electroporation to transform bacteria with the USER enzyme product. The USER enzyme reaction and transformation that successfully produced a clone of pTCVNF1 differed from previous attempts in two distinct ways. DNA ligase was used on the USER reaction, and twice as much competent TOP10F' cells were combined with 11 μ L of the USER reaction solution instead of 2 μ L. The use of DNA ligase with the previous transformation method was not successful. A possible explanation for the success of the method used is that transforming with larger amounts of the USER reaction overcame the low efficiency of the reaction.

Two REDAccuTaq LA PCRs had been planned to eliminate each potential translation start site on pTCp7 Δ p8 that was altered in the p7 cassette, but multiple PCR attempts using a variety of conditions failed to give the intended product. The primers or inserted DNA sequence were likely the cause of this problem, as the PCR was otherwise

similar to the one that created pT1D1ΔLΔp8. The inserted DNA sequence alternated GC-rich and AT-rich regions, and the primers used had annealing temperatures above 60° C. When one of the pairs of primers were used separately with p7 USER primers and annealing temperatures of 55° C to test the individual primers in the pair, the expected short PCR products were reliably produced. Possible explanations for this result are that the DNA polymerase was able to unwind the GC-rich regions when primed by the p7 USER primers, and replicate ssDNA that the other primer was then capable of annealing to, or that the primers are binding to each other in a way that interferes with PCR. After multiple failed PCRs, the plan was changed to use a cassette that already contained the mutations as a template for the p7 insert PCR.

The reason for the GC-rich regions in the CarMV p7 gene is likely that they are part of the 1.7 kb sgRNA promoter (Wang & Simon, 1997). In the ssRNA, the promoter is likely to form complex secondary structure, and it may be possible that the DNA copy of the promoter forms secondary structure when it anneals in PCR. The GC-content of the region being altered with PCR and the presence of secondary structure in the region in the source ssRNA are factors that should be considered in PCR mutagenesis of RNA viruses.

4.2 Possible Further Mutations

It is possible that further modifications will need to be made to pTCVNF1 to create a TCV mutant that is capable of infection. The p7 translational start site is more similar to the Kozak consensus sequence than the p8 start site, which may result in lower expression of TCV p9. Also, an untranslated region 5' of p7 in CarMV was included in

the insert that was formerly upstream of the CarMV 1.7 kb sgRNA. This region includes one AUG that is not a start site in CarMV, but it is now the first AUG on the 1.7 kb sgRNA. Although translation initiation at this site is likely to be practically nonexistent because of lack of similarity with the Kozak consensus sequence, there is still a possibility that the length of the untranslated region will affect translation efficiency of p7 in the mutant TCV.

The untranslated region also contains 40 bases of the 3' half of the 1.7 kb sgRNA promoter of CarMV. Wang & Simon (1997) found that the TCV 1.7 kb sgRNA promoter caused transcription of a sgRNA at low level *in vivo* when as little of the promoter as 10 bases upstream of the transcription start site remained. Therefore, the remaining sequence of the CarMV 1.7 kb sgRNA promoter may cause transcription of a second 1.7 kb sgRNA. Whether this will affect viral infection is unclear, as it depends on the importance of the relative amount of each protein produced.

4.3 Future Research

The mutant TCV will be used to study hypersensitive response suppression in *A. thaliana*. Di-17 *A. thaliana* will be inoculated with the mutant or wild-type virus, and two weeks after inoculation, the plants with systemic infection will be compared for each virus and visually scored for the presence of a systemic hypersensitive response. If systemic HR is observed in *A. thaliana* infected with the mutant virus, that will be evidence for p8 having a role in the suppression of HR.

It is possible that the mutant virus is not capable of infection in *A. thaliana*, as CarMV does not infect *A. thaliana* (Cartwright, 2006), and the movement proteins may

be responsible for host specificity. If that is the case, suppression of hypersensitive response in *N. benthamiana* can be studied by using the coinfiltration of agrobacterium carrying Avr and AvrPto (Jyoti, 2007). The coinfiltration causes transient expression of proteins necessary to trigger a HR in *N. benthamiana*, and Jyoti showed that TCV infection suppressed this HR. Performing the same experiment with the mutant TCV will provide an alternative test for whether p8 is required for suppression of hypersensitive response.

Other potential research uses for the mutant virus is the study of the role of movement proteins in infection phenotype. CarMV, TCV wild-type, and the mutant TCV could be inoculated into a common host, such as *N. benthamiana*, and visual, cellular, and biochemical observations could be made to compare the effects of infection on plants. This could lead to the discovery of specific pathogenic effects caused by p7 and p8.

If p8 is responsible for the suppression of hypersensitive response, the mutant virus could be used for mutational analysis of p8 to determine how it suppresses the HR. The mutant virus expresses p7, which could complement defects in cell-to-cell and systemic movement in mutant p8 genes expressed in transgenic plants. This would allow mutational analysis of p8 *in vivo* which could be screened for any mutations that cause loss of HR suppression.

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