RECOMBINATION OF TWO RNA VIRUSES: *RED CLOVER NECROTIC MOSAIC VIRUS* AND *CARNATION RINGSPOT VIRUS*

By

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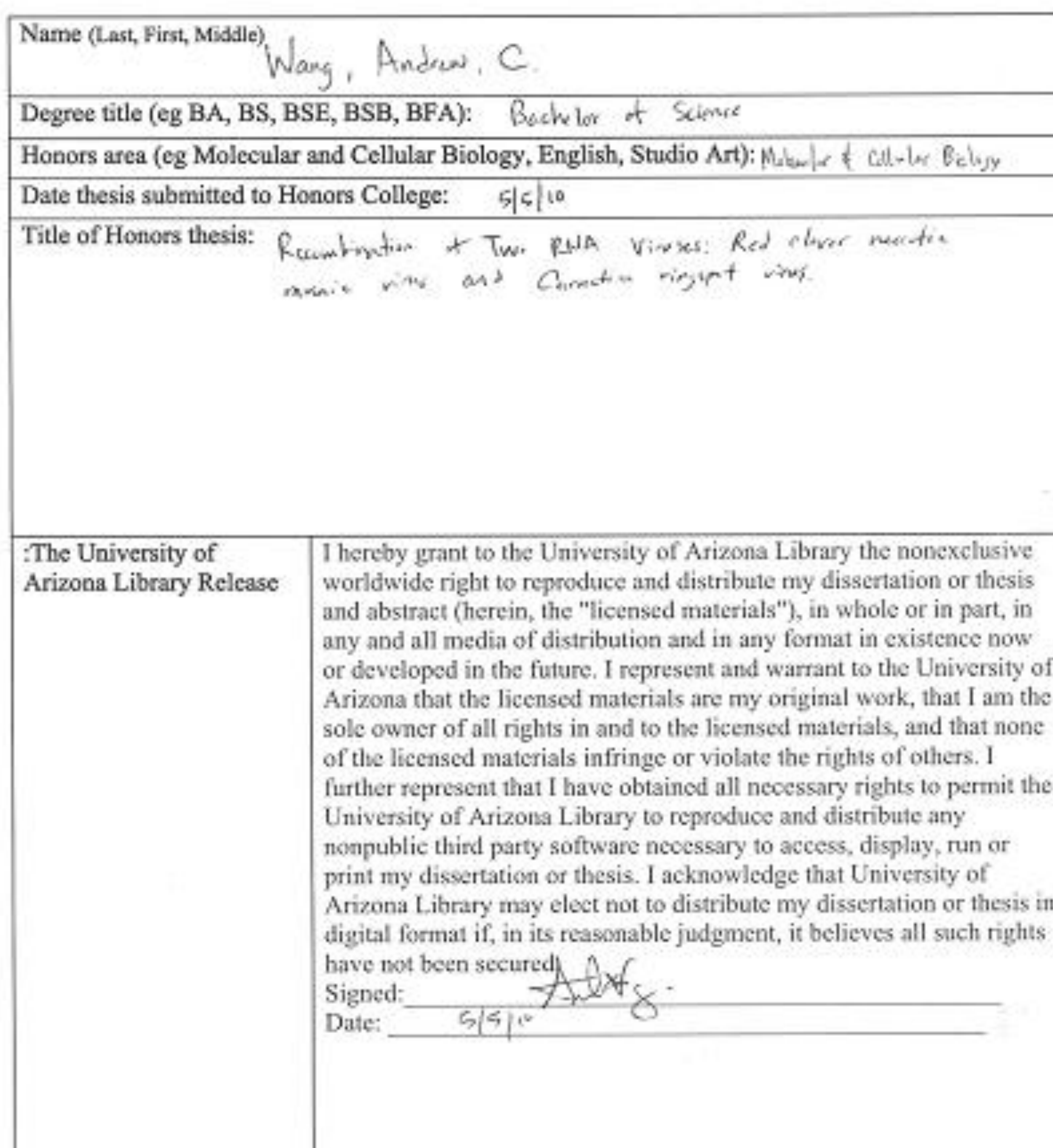

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Abstract

In this research project, two plant RNA viruses, *Red clover necrotic mosaic virus* (RCNMV) and *Carnation Ringspot Virus* (CRSV) were studied and two recombinants were created using RNA1 of RCNMV and RNA2 of CRSV (R1+C2) for one recombinant and RNA2 of RCNMV and RNA1 of CRSV (C1+R2) for the other recombinant. *Nicotiana clevelandii* and *Nicotiana benthamiana* were inoculated with the wild types and the two recombinants. The tissues of the plants were extracted for total RNA and a reverse transcription polymerase chain reaction was performed on the total RNA. Using specific primers, the RCNMV wild type from the 12/1/09 total RNA extraction showed the presence of both R1 and R2, while the CRSV wild type from the 12/1/09 total RNA extraction also showed the presence of both C1 and C2. The recombinant C1+R2 from the 12/8/09 total RNA extraction showed the presence of both C1 and R2 and the recombinant of R1+C2 from the 12/1/09 total RNA extraction also showed the presence of both R1 and C2. The next step in this study would be to clone the terminal ends to find out where exactly recombination occurs.

Introduction

Red clover necrotic mosaic virus (RCNMV) is plant virus that is sap-transmissible to a broad range of herbaceous plant species. It is mainly found in Czechoslovakia, Poland, Sweden, and Britain. RCNMV is a member of the genus *Dianthovirus,* part of the family *Tombusviridae*. Its genome consists of two single stranded RNAs; RNA-1 (4.0kb) and RNA-2 (1.4kb). Both RNA strands lack a 5' cap and a 3' poly A tail. RNA1 consists of many important components such as a replicase component, 27-kDa protein (p27), an N-terminal frame shift product, 88-kDa protein (p88), which contains the RNA-dependent RNA polymerase motif, and a 37-kDa coat protein (CP), which is transcribed as the result of interactions between RNA1 and RNA2 (5). RNA2 consists of a 35-kDa movement protein (MP), which is responsible for viral cell to cell movement in plants. RNA1 contains a translation enhancer element (3' TE-DR1) in the 3' UTR, which can replace the function of the 5'cap (6). RNA2, however, does not contain these translation enhancer elements. Instead, the cap-independent translation activity is tied to the replication of RNA2 (7). Essentially, RNA elements needed to replicate RNA are also keys for cap-independent translation in RNA2. These elements are found in the 5' and 3'UTRs. A 3' stem-loop (SL) structure in the 3'UTR of RNA1 has been found to be important for replication and negative-strand RNA synthesis in RNA2. Because most viral RNA genomes lack a 5' cap, the 3' UTR takes its role for certain translation elements. The 3'UTR is important for the translation and replication in most RNA viruses (4, 7).

Carnation Ringspot Virus (CRSV) is a plant virus found in carnations, grapevines, and orchard crops in Central and Eastern Europe. It is mechanically transmitted through the soil without a biological vector. CRSV is also a member of the genus *Dianthovirus*. Similar to RCNMV, the CRSV genome consists of two nonhomologous, positive-sense, single-stranded

RNAs; RNA1 (3.9kb) and RNA2 (1.5kb). Both RNAs are encapsidated by the 37-kDa capsid protein (CP) (1). The 5’ open reading frame (ORF) codes for p27 and p88, which the CP is expressed from the sub genomic RNA, much like RNA1 of RCNMV. RNA1 of CRSV does contain an additional 3’-terminal open reading frame (ORF), which RCNMV RNA1 lacks (2). The 54K protein contains the RNA-dependent RNA polymerase motif. Much like RCNMV RNA2, CRSV RNA2 is also monocistronic and encodes the 35-kDa MP to help with cell to cell movement (3).

In this study, two recombinants were created between RCNMV and CRSV. One recombinant consisted of RCNMV RNA1 (R1) and CRSV RNA2 (C2), while the other recombinant was RCNMV RNA2 (R2) and CRSV RNA1 (C1). *Nicotiana clevelandii* and *Nicotiana benthamiana* plants were both inoculated with wild type R1R2, wild type C1C2, recombinant R1C2, and recombinant C1R2. The infected leaves were harvested and extracted for total RNA and viral RNA. Using a reverse transcription polymerase chain reaction, segments were amplified to find the presence of the recombinant RNAs and to confirm the recombination. The purpose of this experiment is to determine where recombination occurs between the two RNAs.

Methods

Ligation

In order to undergo transformation, Ligation of digested pCRSV 1.30A and 2.20A cDNA clones by HindIII was first performed to circularize the DNA. First, 32ul of H_2O, 4ul of Biolab 10x T4 ligase buffer, and 4ul of T4 DNA ligase were added in order respectfully. They were mixed together by tapping the tube and then centrifuged briefly. Ten ul of the mix is separated equally into four tubes and 1ul of digested DNA is put into each tube. One set of tubes (pCRSV 1.30A and pCRSV 2.20A) was left at room temperature for 10 minutes and another set of the two tubes was left at room temperature for one hour.

Transformation

To introduce foreign DNA into the circularized DNA, a transformation was then performed. Along with the 4 ligated DNA samples, a positive control (pRC2IG54) and negative control (H_2O) were also used. A 1:1000 dilution of pRC2IG54 to 1000ul H_2O was made and 60ul of bacteria was added to each of the 6 tubes (competent cells were already prepared earlier). The ligated DNA was added to each tube (one ligated DNA for one tube). The samples are incubated on ice for 30 minutes to prepare the cell to become permeable to plasmid DNA. The tubes are then heat shocked at 42˚C in a water bath for 90 seconds to allow the foreign DNA to enter the bacteria. The tubes are then returned to ice for 4 minutes. 600ul of liquid LB is added to each tube and the tubes are incubated again at 37˚C water bath for one hour.

In order to grow a colony of the new transformed bacteria, ampicillin resistant plates were first made. 25ul of ampicillin and 100ul LB were spread evenly to each plate with a glass rod spreader. Spread 300ul of the newly transformed bacteria evenly across the plate making

sure to use the Bunsen Burner to sterilize the spreader after each solution. Incubate the plates upside at 37˚C overnight and then stored at 4˚C.

Mini-preparation

Solution 1 – 50mM glucose, 10mM EDTA, 25mM Tris, pH8.0

Solution 2 – 0.2N NaOH, 1% SDS (solution 2 must be made fresh each time)

Solution 3 – 3/5M KAc

In order to extract plasmid DNA from the bacterial cell, a plasmid mini-preparation was performed next. Before doing so however, we had to perform an inoculation. 2mL of LB was added to four tubes. They were labeled 2.20A RT10 #1, 2.20A RT10 #2, 1.30A RT60 #1, and 1.30A RT60 #2 respectfully (These bacteria were chosen because they showed the most growth on the colonies from the previous experiment). 2 ampicillin resistant plates were made along with 8mL of LB containing 75mg/ml of ampicillin. 12ul of ampicillin was added to 8mL of LB and mixed well. The plates were separated in half by marking the bottom of the plate with a permanent maker. 1.8mL (900ul x 2) of LB was added to each tube. Using the inoculation hook, bacteria colonies that are well isolated were selected. The method of streaking is now used (3 different streaks with 3 different patterns). After each streak, the hook was washed inside the tube labeled with the corresponding number. This was repeated for each of the halves of the plates. The plates were then Incubate overnight at 37 while the four tubes were cultured in a shaker machine.

Mini-preparation was then performed to extract plasmid DNA from the bacterial cell. 1.5mL of the inoculated colony is poured into microfuge tubes. In order to perform mini-preparation, Solution I, II, and III were made earlier in advance. Microfuge for one minute and

the liquid medium is poured off. The pellet was then resuspended in solution 1 by vigorous vortexting. The tube is placed at room temperature for 5 minutes and then 200ul of solution II is added. The tube is inverted many times and then placed in ice for 5 minutes. 150ul of solution III is added and inverted a couple of times before placing on ice for 10 minutes. The tubes are microfuged at top speed for 2.5 minutes and poured into a new tube. An equal volume (~450ul) of phenol:chloroform:isoamyl alcohol (25:24:1 premixed solution) was then added to the new tube as well. The tubes were vortexed vigorously for 10 seconds and microfuged for 5 minutes. 400ul of the top layer was taken and added to a new sterilized tube along with 800ul of ice-cold 95% ethanol. The tubes were mixed by inverting and then placed at room temperature for 5 minutes. They were then microfuged for 5 minutes and the liquid was poured off. The DNA pellet remaining was washed with 70% ethanol, air dried, and then resuspended in 50ul of sterile water containing 20ug/ml RNase A.

Mid-Scale Plasmid Preparation

10g of LB is added with 400ml of ddH_2O, mixed, and heated to boil in a 1L flask. ddH_2O is then added for a final volume of 500ml. Ampicillin is added once the liquid medium has cooled. Each one of the colonies made is then added to a flask and put overnight in a 37˚C shaker. Each flask is poured into a centrifuge bottle the next day and the bottles are balanced and centrifuged at 5000RPM, 4˚C, in JA14. The aqueous phase is then poured into a flask and 4ml of Solution I is added and vortexed. 8ml of solution 2 are added and the tube is mixed gently and then placed in ice. 6ml of solution 3 is added and mixed gently as well. The tube was then centrifuged for 15 minutes at 12000RPM, 4˚C, in JA14. The liquid is then poured into 2 new

centrifuge bottles. 50ml of RNAse is added and the bottles are incubated for 25 minutes and then put in the freezer overnight.

The next day, the solution is thawed in 37˚C and 15ml of p:c:i is added to the bottles and vortexed until the liquid turns white. The bottles are balanced and centrifuged for 5 minutes at 10,000RPM, 4˚C, in JA-17. The extraction is repeated again with the aqueous layer. 14ml of the aqueous phase is transferred to a new tube and 2X the volume of ethanol is added. The tubes are put in -20˚C for 20 minutes, centrifuged, and the liquid is dumped out. 0.82ml of H_2O is added and transferred to a 2.2ml tube. 800ul is transferred to the new tubes leaving 20ul of the solution behind. 0.2ml of NaCl and 1ml of 13% PEG are added and the solution is frozen for one hour. The liquid is then poured out and ethanol is used to wash the tube. The tubes are centrifuged and the ethanol is poured out. The tubes are then air dried for 5 minutes. 500ul ddH_2O are added, the solution is resuspended, and the tubes are put in the freezer.

In Vitro Transcription

To make *in Vitro* infectious transcripts of RCNMV and CRSV RNA-1 and RNA-2, the infectious cDNA clone were first linearized by digestion with restriction enzyme SmaI and HindIII, respectively. The digestion was already completed for RCNMV therefore, all I had to perform was the digestion of CRSV. 110ul of H_2O and 80ul H_2O were added to two separate tubes. 15ul of 10x buffer (red) was added to each tube. 20ul of 1.30A and 50ul of 2.20A were added to the tubes in the same order as step #1. 5ul of HindIII was added to each tube. The tubes were incubated at 37˚C for 2 hours. 3ul of 0.5M EDTA was added to each tube. 150ul p:c:I was also added to each tube and vortexed for 10 seconds and centrifuged for 5 minutes. The top aqueous layer (~150ul) was taken and transferred to a new tube. 1/10 volume of 3M NaAc and 2

volumes (~300ul) of 100% ethanol were added. The tubes were mixed well and kept at -20˚C for one hour or longer.

For *in Vitro* transcription, 4 tubes were labeled pCRSV 2.20A – HindIII, pCRSV 1.30A – HindIII, pRC1IG69 – SmaI, and pRC2IG54 – SmaI. 9.3ul of ddH_2O, 1.0ul DTT, 4ul of 5mM NTP, 4ul of 5X T7/T3 Buffer, 0.2ul of RNAse Inhibitor were each added to each tube. 1.0ul of DNA was added to the corresponding tube with its label. 0.50ul of T7 RNA Pol was then added to each tube. The tubes were incubated at 37˚C water for 90 minutes and at 60 minutes, a 1% gel was run with 1ul of the each sample.

Inoculation

Inoculation preparation involved recombination of the newly transcribed RNA. 9.5ul of pRC1IG69 (R1) was added to 9.5ul of pCRSV2.20A (C2). The same procedure was performed for pRC2IG54 (R2) + pCRSV1.30A (C1) as well as the positive controls R1+R2 and C1+C2. The RNA is now ready for inoculation on plant leaves.

Total RNA Extraction

1.7ml phenol-safe tubes were obtained and labeled. 0.10g of the infected tissue is rolled up such that it can be slid into the tube. The tubes are filled with liquid nitrogen and the tissues are grinded to a fine powder. 0.5ml of TRIZOL reagent is added to the tube to resuspend the powder and the tubes are left on ice for 10 minutes. 100ul of chloroform is added to each tube, the tubes are shaken vigorously and then microfuged for 8min at 13,500rpm. The supernatant is transferred to a new 1.7ml phenol-safe tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) is added. The tube is shaken again and microfuged for 8 minutes at 13,500rpm.

The supernatant is transferred to a new 1.7ml phenol-safe tube for the last time and an equal volume isopropyl alcohol well be added as well. The tube is mixed well by inverting, left on ice for 10 minutes, and microfuged at 13,500rpm for 10 minutes. The RNA will be collected in a pellet at the bottom of the tube. The liquid is poured out and the pellet is washed with 70% ethanol. The tubes are briefly centrifuged and the ethanol is poured out. The pellet is then air-dried in a fume hood for 5 minutes and resuspended in 50ul ddH_2O. The samples of RNA are stored at -20˚C.

Virus Purification

15g of tissue was grinded up in extraction buffer using a mortar and pestle and β Mercapthoethanol was added. The grinded liquid is passed through 4 layers of cheese cloth into a centrifuge tube and the tube is put on ice for 10 minutes. The tube is centrifuged at 10,000RPM in a JA-17 rotor for 10 minutes. PEG, NaCl, and Triton X-100 is added and stirred in a 4˚C water bath for 45 minutes. The solution is centrifuged again at 10,000RPM in a JA-17 rotor for 10 minutes. The supernatant is discarded as much as possible and the remaining supernatant is removed with a pipetteman. Resuspend the pellet in tris-citrate buffer (1/10 of original volume) and triton x-100 is added as well. The solution is stirred in a 4˚C water bath for 45 minutes to resuspend completely and refrigerated.

The virus prep is resuspended by centrifuging the tubes at 10K RPM for 10' in JA-17. The supernatant is transferred to a new centrifuge tube. The original tube with the pellet is resuspended with 0.5ml tris-citrate buffer and refrigerated. 1.89ml of 24% PEG and 0.27ml of 4M NaCl are added to the supernatant tube and incubated on ice for 30 minutes. The tube is then

centrifuged at 10K RPM for 10 minutes in JA-17, the liquid is removed completely with a Pipetman and the pellet is resuspended in 0.5ml tris-citrate buffer.

Viral RNA Extraction

5ul 10% SDS and 12.5ul of 10ug/ml proteinase K is added to each tube from virus prep tube. The tubes are incubated at 37˚C for 10 minutes. 100ul of 10% SDS is added to each tube and incubated at 60˚C for 2 minutes and then put on ice for 3 minutes. 0.6ml of p:c:I (25:24:1) are added and mixed for one minute and then centrifuged for 5 minutes at 13,500RPM. 250ul of the aqueous phase is transferred to a new tube and 250ul of p:c:I is added and centrifuged for 5 minutes at 13,500RPM. 200ul of the aqueous phase is transferred to a clean tube and 1/10 the volume of NH_4Acetate (~20ul) is added along with 2x volumes (400ul) of 100% Ethanol. The solution is mixed well and incubated at -20˚C for 30 minutes. The tubes are then removed from the freezer and centrifuged at 13,500RPM for 20 minutes. The supernatant is poured out and the rest of the liquid is removed with a Pipetman. 1mL of EtOH is added and the tube is inverted and then centrifuged for 1 minute. The liquid is then poured out, the rest of the liquid is removed with a Pipetman, and the pellet is air dried for 5 minutes. The pellet is resuspended in 50ul ddH_2O.

Reverse Transcription Polymerase Chain Reaction

5X First strand (FS) buffer and 0.1M DTT is thawed in 37˚C. 3.75ul of ddH_2O, 1.0ul of 10uM complementary primer, and 1.0ul of total RNA is added to a 0.6ml test tube. The tube is mixed and pulse centrifuged. The tube is then incubated at 70˚C for 5 minutes and chilled on ice for 3 minutes. 2ul 5X FS buffer (invitrogen), 1.0ul DTT 0.1M, 0.25ul superscript III RT

(invitrogen), and 1ul 5uM dNTPs are added to the tube. The tube is again mixed and pulse centrifuged. The tube is incubated at 50˚C for 60 minutes and then incubated at 70˚C for 15 minutes. 40ul ddH_2O is added to dilute the newly synthesized cDNA. Take new 0.5ml PCR tubes and add 1.25ul 10x choice Denville Buffer, 0.25ul identical primer, 0.25ul complementary primer, 0.50 5uM dNTPs, 9ul ddH_2O (make sure you add this first), 0.25ul Denville Choice Taq, and 1ul of the cDNA. The test tube is mixed and pulse centrifuged. The tubes are placed into a PCR machine and the program RC1P27* was run.

* RC1P27

1 = 94˚C for 2 minutes

2 = 94˚C for 45 seconds

3 = 54˚C for 45 seconds

4 = 72˚C for 1 minute

30 cycles total

Then, 5 = 72˚C for 10 minutes

6 = 25˚C forever

Gel Electrophoresis

Weigh out 0.36g of Agarose and add to 40mL of 1X TAE. The solution is mixed and heated on a hot plate until boiling. The mixture is then cooled in water and 2.5ul of 10ug/ml EtBr is added. The solution is then poured into a gel box and let to dry for 20 minutes. The gel is now ready to be loaded. For total RNA extractions, 2ul of RNA is added with 2ul of loading gel. For RT-PCR, 4ul of the cDNA is added with 2ul of loading gel.

Results

A ligation was performed to circularize two digested CRSV cDNA clones: pCRSV1.30A and pCRSV 2.20A (A ligation was not necessary for pRC1IG69 and pRC2IG54, RCNMV cDNA clones). The ligation was done under two conditions; room temperature for 10 minutes and room temperature for 60 minutes. After ligation, a transformation was performed with competent bacterial cells and then spread onto ampicillin resistance LB growth plates. The results of the transformation showed lots of bacterial growth on pCRSV1.30 RT10 and some bacterial growth on pCRSV1.30A RT60. pCRSV2.20A RT10 showed some bacterial growth and pCRSV2.20 RT60 showed the most bacterial growth. As expected, the positive control (pRC2IG54) showed some bacterial growth, while the negative control (ddH_2O) showed no bacterial growth.

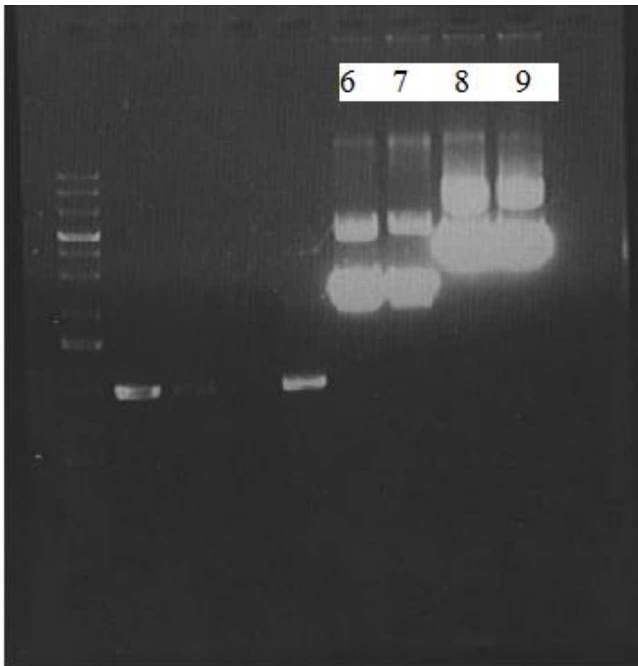


Figure 1 - 0.8% agarose gel. Lane 6 and 7 - pCRSV2.20A RT10. Lane 8-9: pCRSV1.30A RT60

After the mini-preparation, gel electrophoresis was run with a 0.8% Agarose gel and the results (figure 1) showed bright defined bands for all four samples. Thus, this shows a large amount of DNA. The samples that were chosen were pCRSV2.20A at room temperature for 10 minutes and pCRSV1.30A at room temperature for 60 minutes.

After the mid-scale plasmid preparation, gel electrophoresis was run with a 0.9% Agarose gel and the results (figure 2) showed bright defined bands for all four samples. However, the two bands that did not have 13% PEG and 4M NaCl seemed to have another band further down the gel.

Figure 2 - 0.8% agarose gel. No ladder used. Lane 1 - pCRSV1.30A with 13%PEG and 4MNaCl. Lane 2 - pCRSV2.20A with 13%PEG and 4MNaCl. Lane 3 - pCRSV1.30 without 13%PEG and 4MNaCl. Lane 4 - pCRSV2.20 without 13%PEG and 4MNaCl

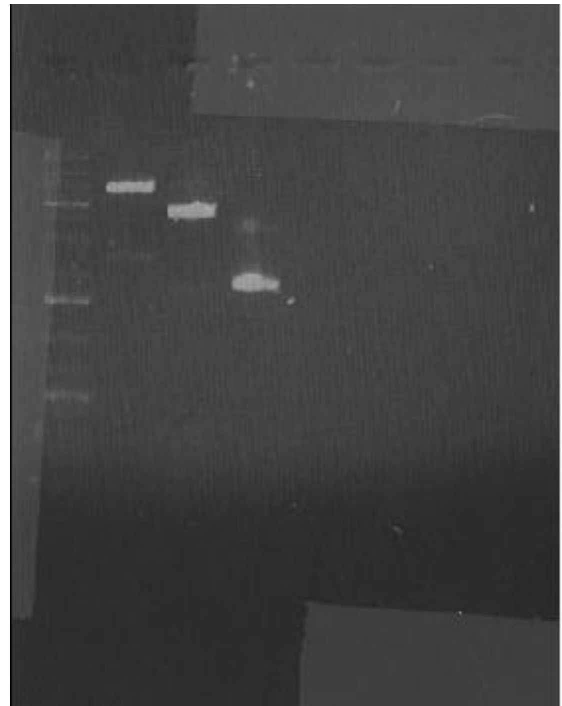


Figure 3 - 0.9% agarose gel. Lane 2 - pCRSV1.30A. Lane 3 - pCRSV2.20A

After digestion of pCRSV 1.30A and 2.20A with HindIII, a 0.9% agarose gel shows the linearized cDNA (figure 3).

An *in Vitro* transcription is performed and a 0.9% agarose gel is ran (figure 4). The first bands represent the cDNA similar to figure 3, while the bands further down the gel represent the newly transcribed RNA. Figure 5 shows the second *in Vitro* transcription performed after the first inoculation did not seem to work.

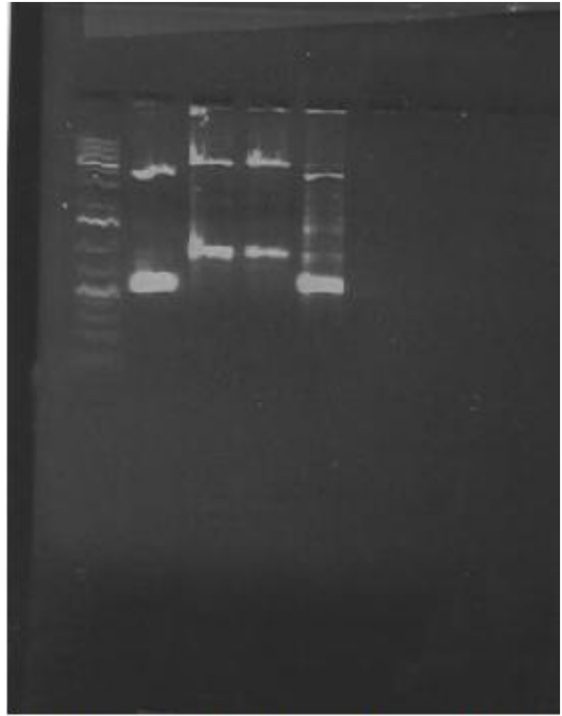


Figure 4 - 0.9% agarose gel. Lane 2 - pCRSV2.20A with HindIII. Lane 3 - pCRSV 1.30A with HindIII. Lane 4 - pRC1IG69 with SmaI. Lane 5 - pRC2IG54 with Sma1

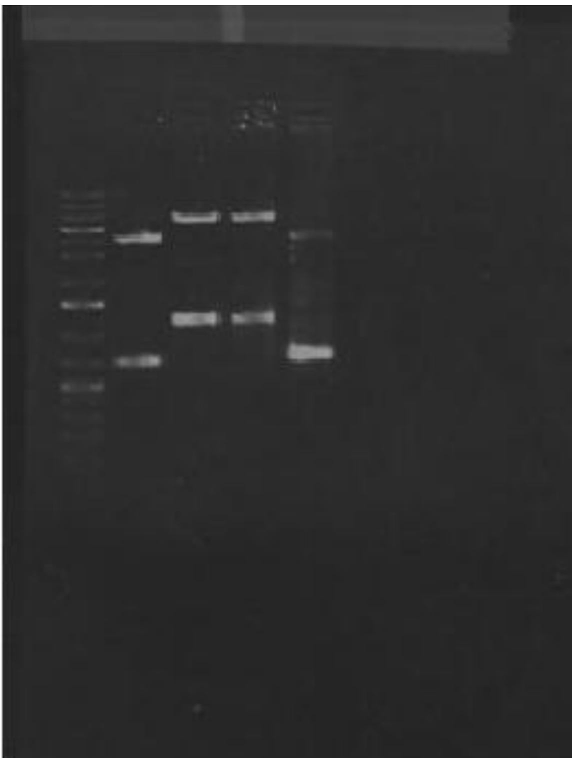


Figure 5 - 0.9% agarose gel of second in vitro transcription. Lane 2- pCRSV2.20A with HindIII. Lane 3 - pCRSV1.30A with HindIII. Lane 4 - pRC1IG69 with Sma1. Lane 5 - pRC2IG54 with SmaI

The first inoculation performed on 10/30/09 used the first *in Vitro* transcription seen from figure 4. The second inoculation performed on 11/6/09 used the second *in Vitro* transcription seen from figure 5.

Inoculation Observations 10/30/09

	Original Leaves Inoculated*	**New Growing Leaves**
R1+R2 (wild type)	Little to no symptoms	Good systemic symptoms
R1+C2	Little to no symptoms	Severe systemic infection
C1+C2 (wild type)	Parts of leaves infected	No symptoms
C1+R2	One leaf infected	No symptoms

Inoculation Observations 11/6/09

	Original Leaves Inoculated*	**New Growing Leaves**
R1+R2 (wild type)	Little to no symptoms	Good systemic Infection
R1+C2	No symptoms	No symptoms
C1+C2 (wild type)	Severe symptoms	Good systemic infection
C1+R2	Symptoms on one leaf	No symptoms

Inoculation Observation 11/20/09

	Original Leaves Inoculated*	**New Growing Leaves**
R1+R2 (wild type)	Severe symptoms	Some systemic infection
R1+C2	Some symptoms	Systemic infection
C1+C2 (wild type)	Some symptoms	Some systemic infection
C1+R2	Some symptoms	Severe systemic infection

*Mature full grown leaves were chosen to be the original inoculated leaves.

Below are pictures of the infected plants from the 10/30/09 inoculations with the R1+R2 wild type, R1+C2 recombinant, C1+C2 wild type, and C1+R2 recombinant. The signs of infection are indicated with the black arrows.

Figure 6 - Nicotiana benthamiana showing symptoms of infection with R1+R2 wild type

Figure 6B - Nicotiana benthamiana showing symptoms of infection with R1+R2 wild type

Figure 7A - Nicotiana benthamiana showing symptoms of infection with R1+C2 recombinant

Figure 7B - Necotiana clevelandii showing symptoms of infection with R1+C2 recombinant

Figure 8A - Necotiana benthamiana shows symptoms of infection with the C1+C2 wild type

Figure 8B - Necotiana benthamiana shows symptoms of infection with the C1+C2 wild type

Figure 9A - Necotiana benthamiana shows symptoms of infection with C1+R2 recombinant

Figure 9B - Necotiana benthamiana showing symptoms of infection with the C1+R2 recombinant

A series of total RNA extractions were performed with 0.10g of an infected leaf, TRIZOL reagent, and Phenol: Chloroform: Isoamyl alcohol (25:24:1). Gel electrophoresis was performed on each one of these extractions with a 0.9% Agarose Gel. Figure 10 shows the first total RNA extraction performed on 12/1/09. The control (lane 2) and R1+C2 (lane 6) show the sharpest band, which means the most RNA was extracted from these leaves. The C1+C2 wild type and the C1+R2 recombinant showed bands, but were not as bright as the R1+R2 wild type and the C2+R1 recombinant.

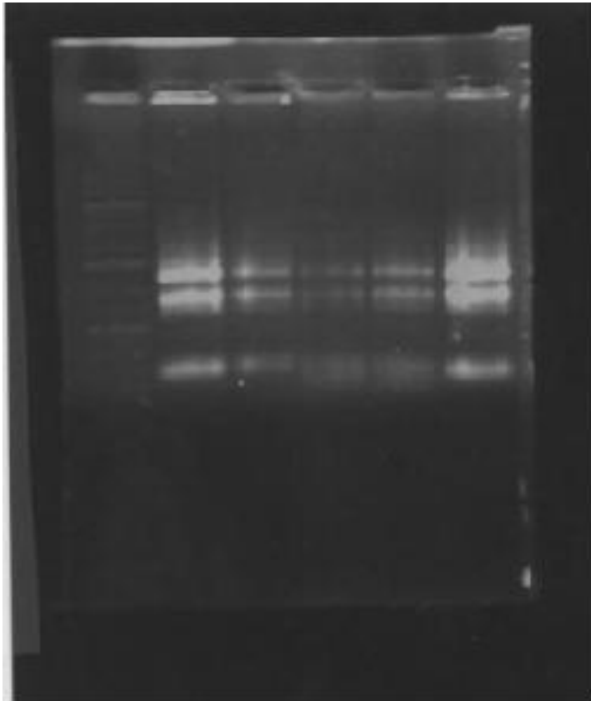


Figure 10 - 0.9% agarose gel of total RNA extraction. Lane 2 - control. Lane 3 - R1+R2. Lane 4 - C1+C2. Lane 5 - C1+R2. Lane 6 - R1+C2.

Figure 11 shows the total RNA extraction for 9 samples performed on 12/8/09. The dates are the inoculation dates:

1. Control from 10/30/09 N. benthamiana
2. R1+R2 from 11/20/09 N. benthamiana
3. R1+C2 from 11/20/09 N. benthamiana
4. C1+R2 from 11/20/09 N. benthamiana
5. C1+C2 from 11/20/09 N. benthamiana
6. R1+R2 from 10/30/09 N. benthamiana
7. R1+R2 from 9/15/09 N. clevelandii
8. R1+C2 from 9/15/09 N. clevelandii
9. C1+R2 from 10/30/09 N. benthamiana

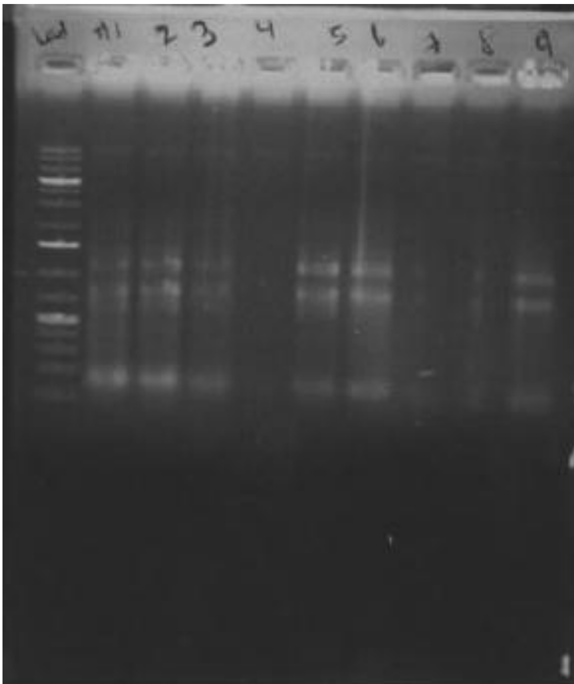


Figure 11 - 0.9% agarose gel of total RNA extraction. #1-6, 9: N. benthamiana, #7 and 8: N. clevelandii.

Figures 12-16 are the rest of the total RNA extractions that was performed.

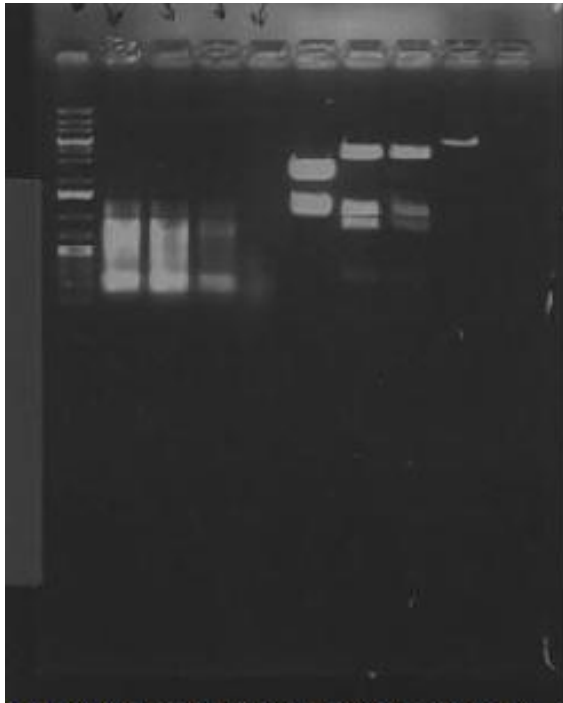


Figure 12 - 0.9% agarose gel of total RNA extraction. Lane 2 - negative control (2/2/10). Lane 3 - negative control (1/28/10). Lane 4 - R1+R2 (2/2/10). Lane 5 - R1+R2 (1/28/10).

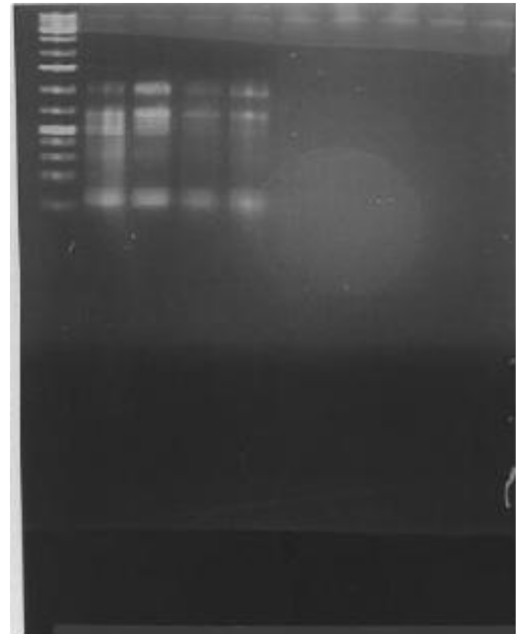


Figure 13 - 1.5% agarose gel of total RNA extraction for 2/4/10 of N. clevelandii. Lane 2 - R1+R2. Lane 3 - R1+C2. Lane 4 - C1+C2. Lane 5 - C1+R2

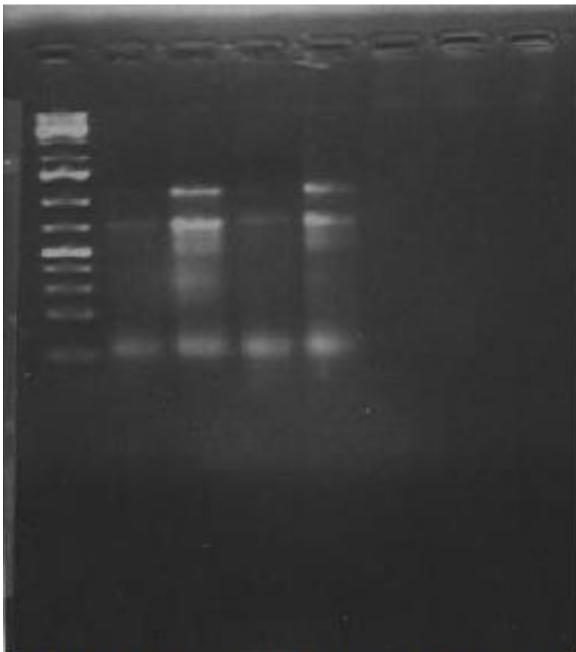


Figure 14 - 0.9% agarose gel of total RNA extraction on 2/9/10 for N. benthamiana. Lane 2 - R1+R2. Lane 3 - R1+C2. Lane 4 - C1+C2. Lane 5 - C1+R2

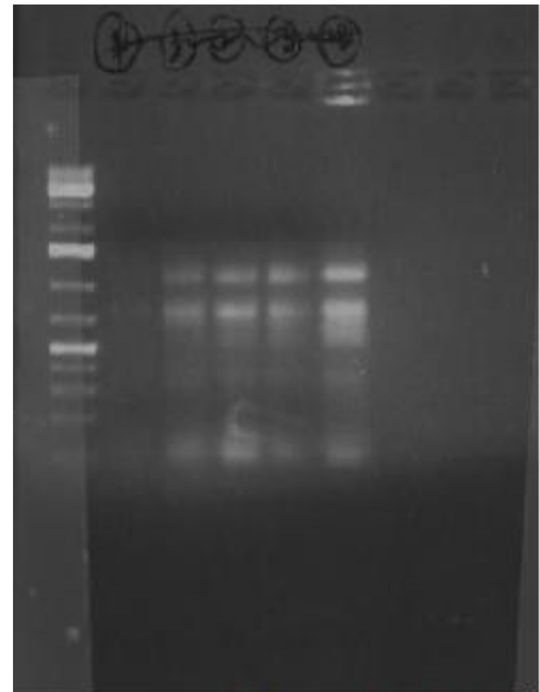


Figure 15 - 1.5% agarose gel of total RNA extraction on 3/9/10 for N. benthamiana. Lane 2 - R1+R2. Lane 3 - R1+C2. Lane 4 - C1+C2. Lane 5 - C1+R2. Lane 6 - Neg control

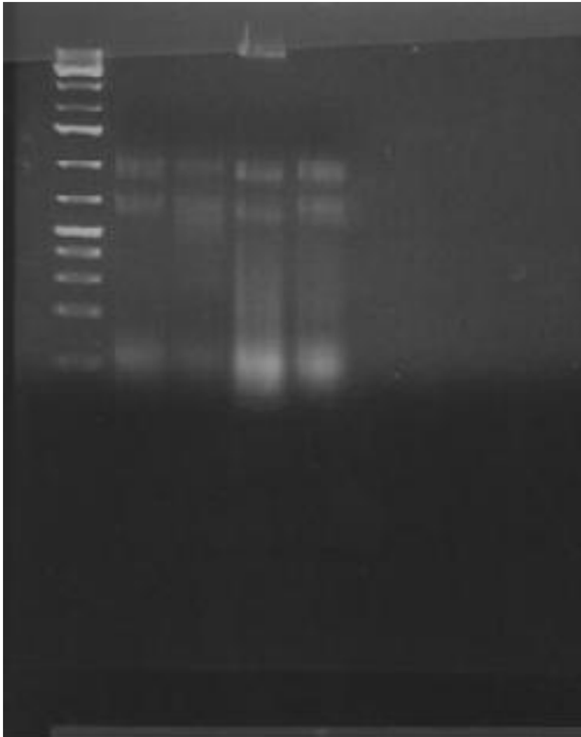


Figure 16 - 0.9% agarose gel of total RNA extraction on 3/24/10 for N. clevelandii. Lane 2 - R1+R2. Lane 3 - R1+C2. Lane 4 - C1+C2. Lane 5 - C1+R2

Two viral purifications were performed with B Mercapthoethanol, Tris-citrate buffer, and Triton-X-100. Gel electrophoresis was performed on each purification with 1.5% Agarose Gel. However, there were no visible bands that were shown (Figures 17-18). As a result, good total RNA extraction products were used instead of virus purification products for a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

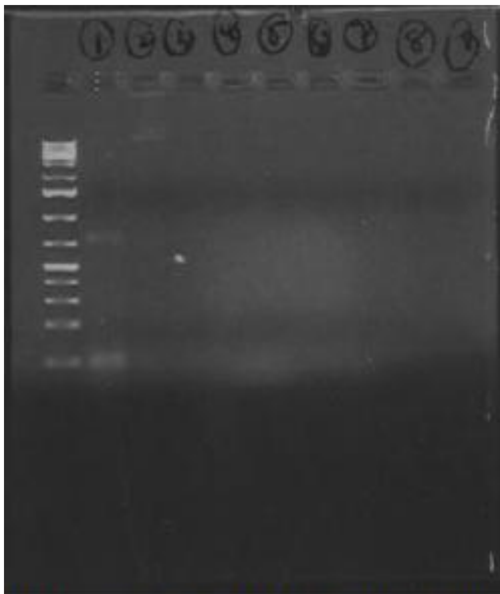


Figure 17 - 1.5% agarose gel of viral RNA extraction on 2/25/10. Only the control (lane 1) showed up on the gel.

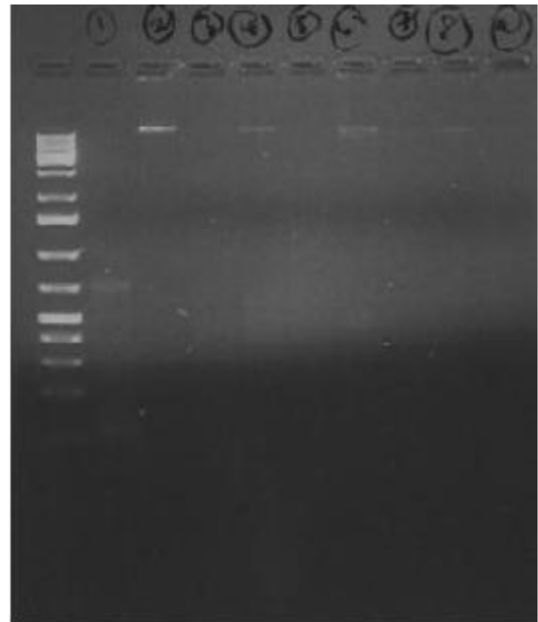


Figure 18 - 1.5% agarose gel of viral RNA extraction on 3/3/10. Again, only the control (lane 1) showed up on the gel

The extracted RNA was used with a selected primer complementary to the RNA strand to undergo reverse transcription using 5X FS RT Buffer Invitrogen and Superscript III RT Invitrogen to create a cDNA. Using the cDNA, a PCR was run with 10x choice Denville Buffer, Denville choice Taq, and the primers needed to amplify the specific region. A 0.9% Agarose Gel is then run to confirm the amplification and presence of the RNA strand we are looking for.

	Identical Strand Primer	**Complementary Strand Primer**
RCNMV, RNA1	1-T7-5'	R1C980*, P830
RCNMV, RNA2	RNA2-5', SL8.16.91A	R2C1220, SL.81.91B
CRSV, RNA1	CRSV1-3100	CRSV1-3500
CRSV, RNA2	CRSV2-700	CRSV2-1100

Table 4 shows a list of the primers used to find the presence of the corresponding RNA

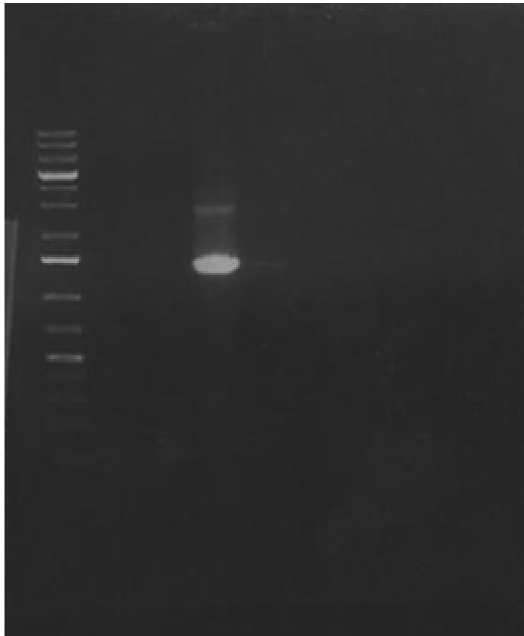
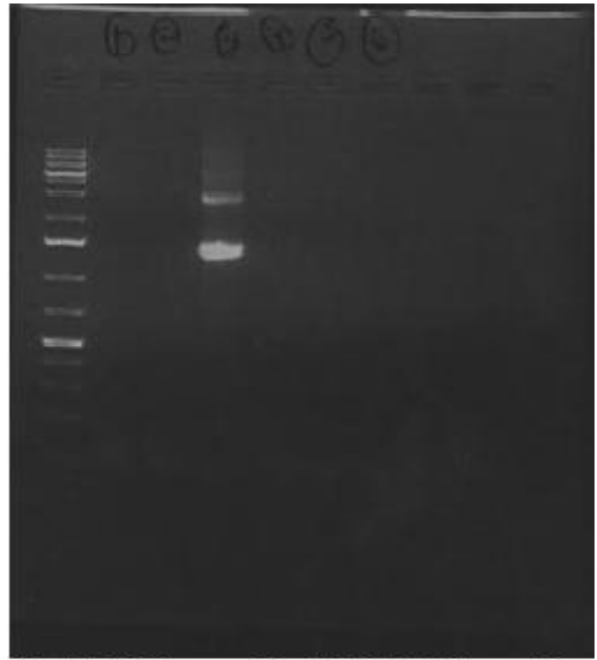


Figure 19 - 0.9%agarose gel from RT-PCR of cDNA created from total RNA (12/1/09) with primers 2-T7-5' and R2C1220. Lane 2 - negative control (ddH2O). Lane 3 - Control. Lane 4 - R1+R2

The RT-PCR performed on 4/2/10 shows the presence of RCNMV RNA2 in R1+R2 (Figure 19). The RT-PCR was repeated again with the same primers on 4/8/10, but this time the total RNA of R1+C2, C1+C2, and C1+R2 from 12/1/09 were also used to find the presence of RCNMV RNA2. However, the gel gave the same results and did not show presence of RCNMV RNA2 for the C1+R2 recombinant (Figure 20).

The RT-PCR performed on 4/12/10 with primers CRSV2-1100 and CRSV2-700 shows the presence of CRSV RNA2 in R2+C2, C1+C2, and a little in C1+R2 (Figure 21).



Figured 20 - 1.5%agarose gel from RT-PCR of cDNA created from total RNA (12/1/09) with primers 2-T7-5' and R2C1220. Only one band showed for R1+R2 and none for C1+R2.

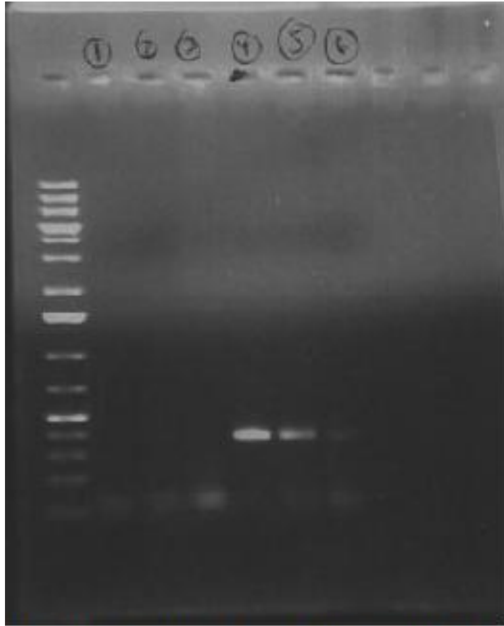


Figure 21-0.9%agarose gel from RT-PCR of cDNA created from total RNA (12/1/09) with primers CRSV2-1100 and CRSV2-700. Bands show up for R1+C2 (lane 4), C1+C2 (lane 5), and C1+R2 (lane 6).

The RT-PCR performed on 4/14/10 with primers R1C980, 1-T7-5', and P830 shows the presence of RCNMV RNA1 in R1+R2 and R1+C2 (Figure 22).

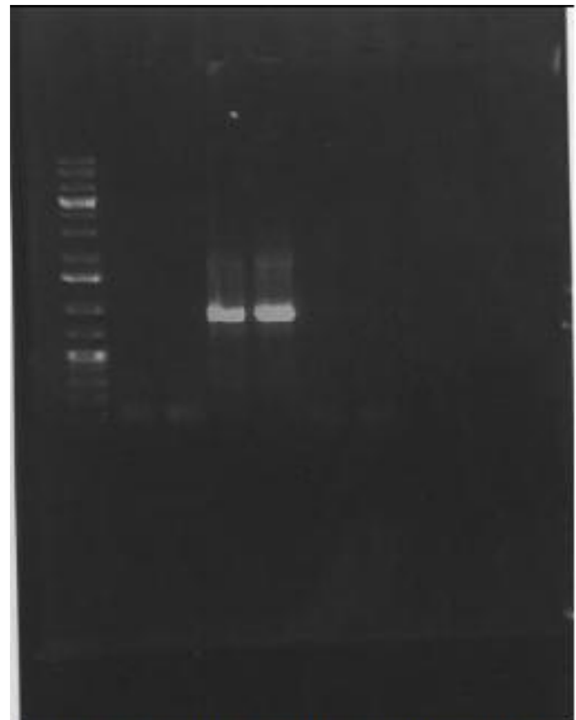


Figure 22 - 0.9%agarose gel from RT-PCR of cDNA created from total RNA (12/1/09) with primers R1C980, 1-T7-5', and P830. Bands show up for R1+R2 and R1+C2

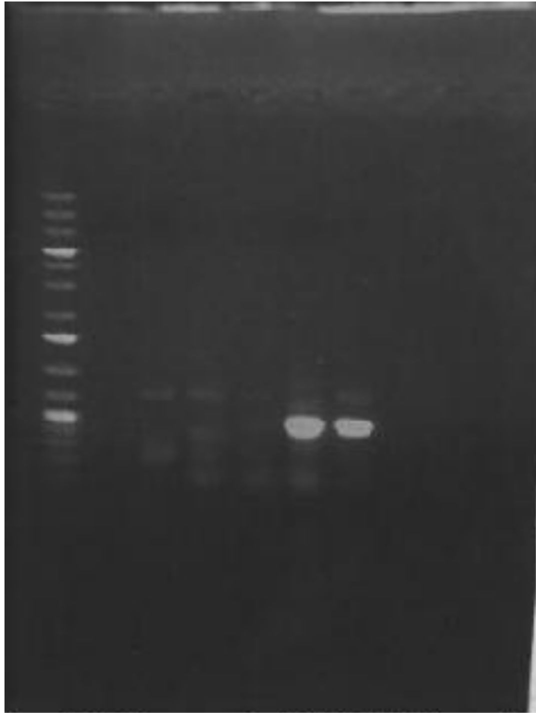


Figure 23 - 0.9%agarose gel from RT-PCR of cDNA created from total RNA (12/1/09) with primers CRSV1-3500 and CRSV1-3100. Bands show up for C1+C2 and C1+R2

The RT-PCR performed on 4/16/10 with primers CRSV1-3500 and CRSV1-3100 shows the presence of CRSV RNA 1 in C1+C2 and C1+R2 (Figure 23).

RT-PCR was performed on 4/21/10 with primers SL.8.16.91A and SL.8.16.91B shows the presence of RCNMV RNA2 in R1+R2 only (Figure 24).

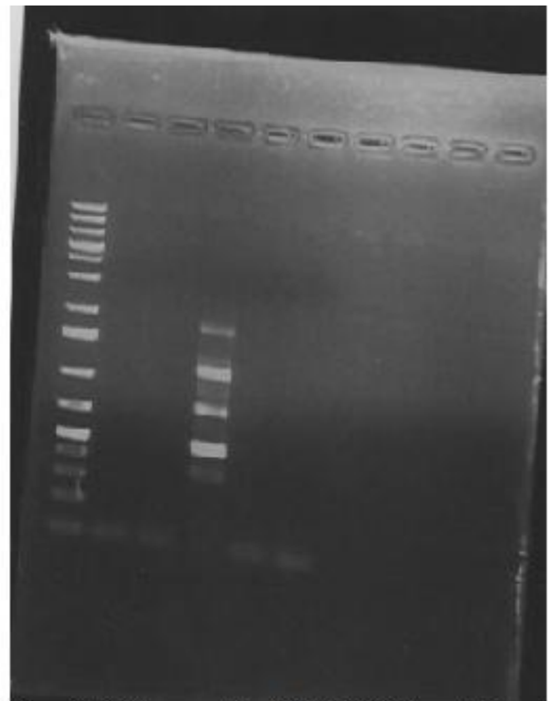


Figure 24 - 0.9%agarose gel from RT-PCR of cDNA created from total RNA (12/1/09) with primers SL.8.16.91A and SL.8.16.91B. Bands only showed up for R1+R2

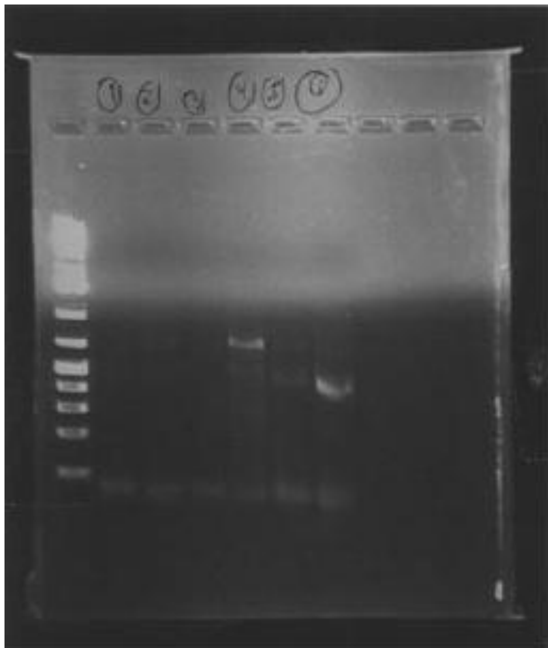


Figure 25 - 0.9%agarose gel from RT-PCR of cDNA created for different total RNAs to find the presence of RCNMV RNA2 in C1+R2. Lane 1 - negative control. Lane 2 - control (12/1/09). Lane 3 - C1+R2 (2/4/10). Lane 4 - C1+R2 (2/9/10). Lane 5 - C1+R2 (3/9/10). Lane 6 - C1 +R2 (12/8/09)

The RT-PCR performed on 4/26/10 with primers SL8.16.91A and SL8.16.91B show the presence of RCNMV RNA2 for C1+R2 (2/9/10) and C1+R2 (12/8/09)

The RT-PCR performed on 4/26/10 with primers CRSV1-3100 and CRSV1-3500 show the presence of CRSV RNA 1 for C1+R2 (12/8/09).

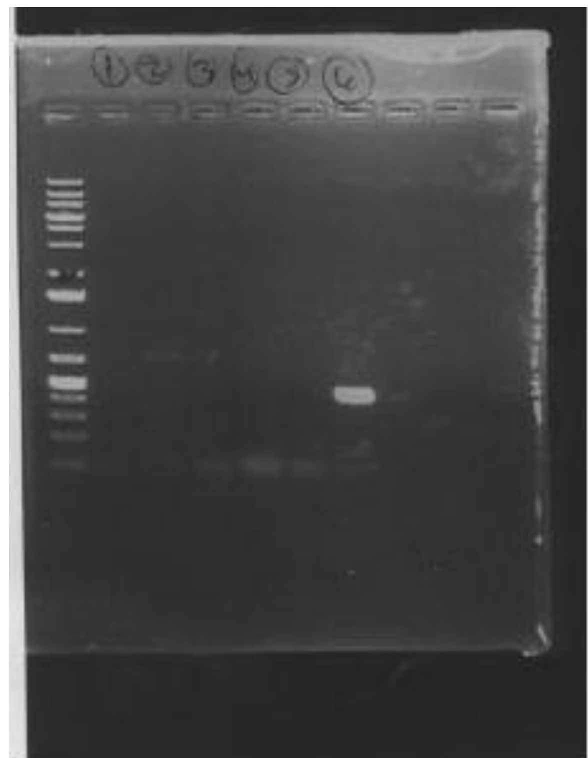


Figure 26 - 0.9%agarose gel from RT-PCR of cDNA created from different total RNAs to find presence of CRSV RNA1 in C1+R2. Lanes are loaded in the same order as Figure 25

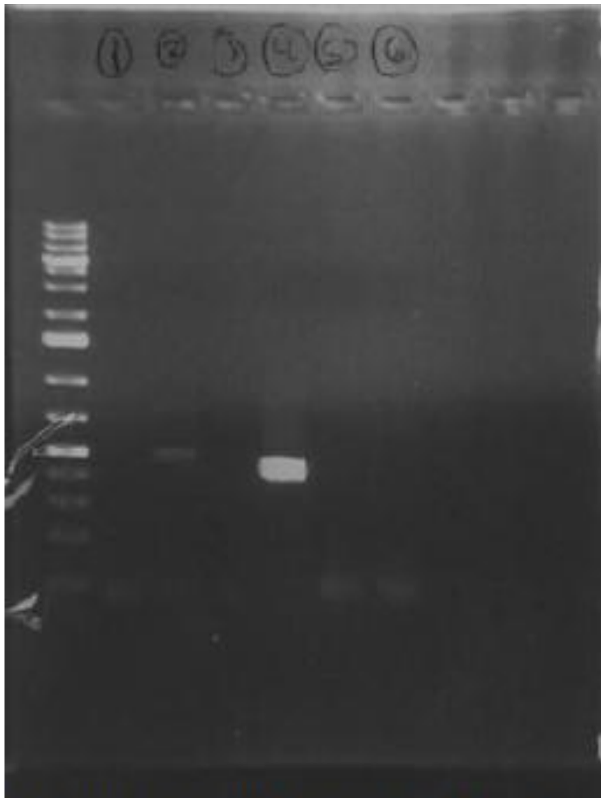


Figure 27 - 0.9%agarose gel from RT-PCR of cDNA clones of C1+R2 (12/8/09) to find the presence of the 5' and 3' end of CRSV RNA1 and 5' and 3' end of RCNMV RNA2.

RT-PCR performed on 4/29/10 was used with three different sets of primers. Lane 2, which used primers SL8.16.91A and SL8.16.91B showed a slight band along with Lane 4, which used primers CRSV1-3100 and CRSV1-3500. Lane 6, which used 2-T7-5' and SL.8.16.91B showed no bands. Lanes 1, 3, and 5 were negative controls.

Discussion

After ligation of the HindIII digested pCRSV1.30A and 2.20A, the transformation showed the presence of the plasmids through the bacterial growth on the LB plates. After the mini-preparation, the 0.8% agarose gel showed great amounts of the plasmid DNA of the expected sizes, proving the transformation successful (figure 1). The mid-scale preparation that was performed also showed a good amount of DNA (figure 2). The two bands that were not added with 13% PEG and 4M NaCl had two extra bands further down the gel. This is due to the fact that PEG and NaCl precipitate only DNA, and because there is no 13% PEG and 4MNaCl, the RNA was not removed so the two extra bands are the left over RNA. Once the plasmid preparation was complete, the digestion with HindIII also proved to be successful after running the products on a 0.9% gel (figure 3).

The *in Vitro* transcription of the cDNA created viral RNA used to prepare the wild type viruses and the recombinant viruses (figure 4). The gel shows 2 bands with the top band representing the linearized plasma DNA and the bottom band represents the newly synthesized RNA. After the first inoculation on 10/30/09 (figures 6-9), R1+C2 recombinant showed the most severe symptoms on the young leaves, which can mean that CRSV RNA 2 has to be present because of the systemic spread of the symptoms. However, the original inoculated leaves only showed a few symptoms, not nearly as severe as the new growing leaves. This makes sense because the new growing leaves are constantly replicating RNA and thus, may play a role in also replicating the viral RNA. On the other hand, the original inoculated leaves have already reached their limit in terms of growth, so there is far less RNA replication. The R1+R2 wild type showed symptoms similar to R1+C2 having good symptoms on the new growing leaves and not as good symptoms on the original inoculated leaves. The R2+C1 recombinant had symptoms similar to

the C1+C2 wild type and only had one original leaf that showed symptoms while none of the younger leaves showed any signs of symptoms. The issue here could have been due to error in inoculation or incomplete in vitro transcription. Therefore, another *in Vitro* transcription was performed and new RNA was obtained to create new recombinants (figure 5). Another inoculation was performed on 11/6/09, which did not prove to be much better than the 10/30/09 inoculation. R1+R2 wild type showed signs of spread, but the symptoms were not as severe. C1+C2 wild type also showed signs of spread, but only to one leaf. The original inoculated leaves however, did show severe symptoms. Both recombinants showed poor results and only had a few leaves with symptoms. From these two inoculations, infected leafs with good symptoms were harvested and grinded up in 400ul of grinding buffer using a mortar and pestle. Using the pestle, new *Nicotiana benthamiana* and *Nicotiana clevelandii* were inoculated on 11/20/09.

This inoculation proved to be much more successful as R1+R2, R1+C2, and C1+R2 all showed signs of symptoms on original and new leaves for *Nicotiana benthamiana.* The C1+C2 wild type however, did not show definitive symptoms. For *Nicotiana clevelandii,* R1+R2 showed severe symptoms on original and new leaves, while C1+R2 and R1+C2 showed symptoms on new leaves and C1+C2 did not have any symptoms. Both types of plants seem to show similar infection patters with R1+R2 wild type being the most infective and C1+C2 being the least infective.

Viral purification and viral RNA extraction allows for a better way to obtain pure viral RNA. However, the procedure to do so can be cumbersome and difficult to obtain good results. After the first viral purification on 2/12/10, the viral RNA extraction was performed using half of the purification samples on 2/22/10. After running a 1.5% agarose gel, there were no signs of

viral RNA shown in the bands (figure 17). Another viral RNA extraction was performed on 2/26/10 using the other half of the purification samples from 2/12/10. Again, there was no sign of viral RNA after running a 1.5% agarose gel. One last viral RNA extraction was performed on 3/10/10 using the viral purification samples from 2/19/10. After running a 1.5% agarose gel, no signs of viral RNA were found again (figure 18). One obvious reason for the lack of results could be due to the instability of RNA. The viral purification samples were stored in a refrigerator for more than a week before being extracted for viral RNA. As a result, the RNA may have degraded within this week. Due to time constraints, it was difficult to perform the viral RNA extractions right after purification. The tissues were harvested on 1/26/10 and stored in a 20 C refrigerator. Thus, time may have played a factor again as the tissues may have degraded before performing viral purification. Of course, the tissues chosen for this procedure may not have contained large amounts of viral RAN at all. A final reason to explain the lack of results could be simple experimental errors or the use of outdated buffers and reagents. However, because the procedure was repeated three times, it is unlikely that the absence of viral RNA is due to experimental errors. On the other hand, some buffers and reagents being used were ten to fifteen years old. With more time, it would be rather simple to fix these factors and successfully extract viral RNA. Due to the lack of time, total RNA extraction seemed to be a better option.

Though total RNA extraction does not separate host RNA from viral RNA, it is a safer and more reliable method. Rather than extracting viral particles directly from the tissues, the total RNA is extracted first. Several total RNA extractions were performed for because the procedure is extremely difficult to perfect and takes lots of practice, attention to detail, and repetition. Also, it is better to obtain as many samples of total RNA as possible to be used later on for reverse transcription polymerase chain reaction. It is important to select tissue with clear symptoms and

signs of infection to ensure that the total RNA being extracted also will contain the viral RNA. The first total RNA extraction performed on 12/1/09 yielded good results. According to figure 10, there is a great amount of total RNA, especially for the control and the R1+C2 recombinant. Many more total RNA extractions were performed following this one to create a good pool of total RNA available. There were some extractions that yielded poor results. These poor results were found to be due to several factors. One of the first steps of extracting total RNA requires grinding up the tissue into a fine powder using liquid nitrogen. This is a very tricky step because the tissue must be grinded immediately and in a specific manner to avoid buildup of frozen tissue at the bottom of the tube. If this technique is not done correctly, there will be tissue that is not grinded and cannot be resuspended with the TRIZOL reagent. An extraction done on 1/26/10 (figure 11) shows the result of the extraction due to the error in the grinding technique. Moreover, during extraction with phenol, chloroform, and isopropyl alcohol (25:24:1), it is key to not touch the organic layer when transferring the supernatant to another tube. As seen in the extraction from 1/28/10 (figure 12), the bands may show signs of degradation if the organic layers are touched or transferred along with the supernatant. Last, once the pellet is formed, an important step is to make sure the pellet is not too dry or too wet with ethanol. During some extractions, the pellet was too dry and could not be resuspended with the ddH_2O. According to the TRIZOL reagent spec sheet, the mixture should be incubated at 60 C for 10 minutes if the resuspension does not work. However, even with the incubation, the pellet still would not fully resuspend with ddH_2O. This affected the loading of the gel as seen in the extraction done on 2/9/10 (figure 14).

Reverse transcription polymerase chain reaction (RT-PCR) was performed next to find the presence of the corresponding RNAs. Figure 19 and figure 20 show the presence of RCNMV

RNA 2 in R1+R2, but in C1+R2 however. This could be due to the fact that the recombination of C1+R2 may have omitted the 5' end of RCNMV RNA2. Figure 21 shows the presence of CRSV RNA 2 in R1+C2, C1+C2, and C1+R2. However, CRSV RNA 2 should not be present in C1+R2. One explanation could be that there was contamination between C1+C2 and C1+R2 either when loading the gel or setting up the PCR. Figure 22 shows the presence of RCNMV RNA 1 in R1+R2 and R1+C2. These results are expected and show that the recombination of R1 and C2 worked out correctly. Figure 23 shows the presence of CRSV RNA 1 in C1+C2 and C1+R2. These results are also expected and show that the recombination of C1 and R2 worked successfully. Figure 24 shows the presence of RCNMV RNA1 in R1+R2, but again, not in C1+R2. Different primers, SL.8.16.91A and SL.8.16.91B (which correspond to nucleotides 161-178 and complementary to nucleotides 643-659, respectively), were used for this RT-PCR instead of the primers, 2-T7-5' and R2C1220 (which look for the presence of the 5' end of RCNMV RNA2). However, there were still no presence of RCNMV RNA2 for C1+R2 showing that maybe R2 is only present starting with nucleotides upstream of nucleotide 659. Given more time for the project, a RT-PCR of C1+R2 would be run to search for the 3' end of RCNMV RNA2.

Figures 25 shows the presence of RCNMV RNA2 in the total RNA extractions from 2/9/10 and 12/8/09. Figure 26 shows the presence of CRSV RNA1 in the total RNA extractions from 12/8/09. Thus C1+R2 from 12/8/09 was then used to find the presence of the 3' and 5' ends of both RCNMV RNA2 and CRSV RNA1. According to figure 27, lane 2 shows the presence of RCNMV RNA2, but the lack of a band on lane 6 show that the 5' of RCNMV RNA2 is still missing. Lane 4 shows a presence of CRSV RNA1, which is what we expected to find. So far in the experiment, it has only been proven that the corresponding RNAs do exist, but the 5' and 3'

ends are not proven to be found. With more time, RT-PCRs would be run to find both 3’ and 5’ ends of both RCNMV RNA2 and CRSVRNA1. The same experiments would be tested for R1+C2 as well. Once these are found, the terminal ends would be cloned to find out exactly where the recombination takes place.

Acknowledgements

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