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Jennifer J. (Jennifer Jeane) Gilbert  
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SATELLITE RNA OF TOBACCO RINGSPOT VIRUS

by

Jennifer J. Gilbert

A Thesis Presented  
to the Faculty of  
the Chemistry Department of  
Western Washington University

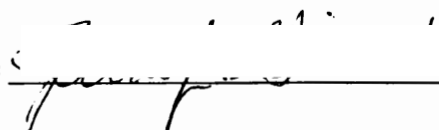
Submitted in Partial Fulfillment  
of the Requirements for the  
Bachelor's Degree with  
Honors in Biochemistry

Supervised by Professor Gerry Prody  
Department of Chemistry  
The College of Arts and Sciences  
Western Washington University

June 2000

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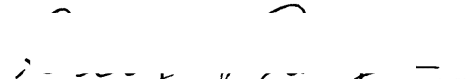
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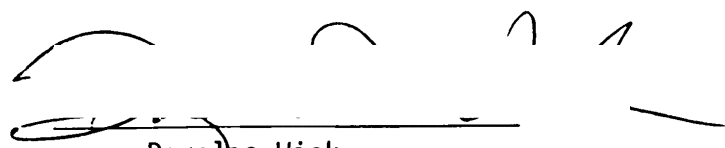
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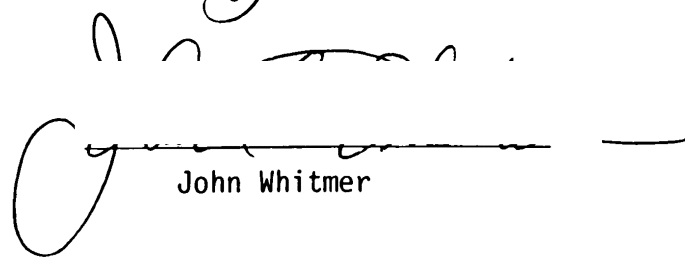
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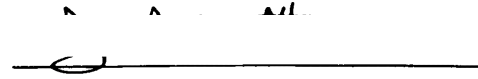
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Bachelor's Degree with  
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## ABSTRACT

The satellite RNA found with tobacco ringspot virus is capable of attenuating the symptoms of viral infection. Although the satellite appears within viral capsids after several passages of infected plants, the origin of this satellite RNA sequence has not been previously determined. Earlier work indicated a potential mitochondrial origin of this sequence. Using satellite specific primers and the polymerase chain reaction (PCR), the mitochondrial DNA of uninfected tobacco cells was probed for sequence corresponding to the satellite RNA. A new protocol was adapted for the isolation of mitochondrial DNA in which specific isolation of protoplasts and mitochondria was not required. Although two PCR products were isolated, sequencing of these products and analysis with the MacVector 3.5 Sequence Analysis program showed no homology with the budblight strain of tobacco ringspot virus satellite RNA.

## **Acknowledgements**

I would like to extend my sincere appreciation to the following individuals:

Thesis Advisor, Dr. Gerry Prody, for her willingness, patience, expertise, and enthusiasm;

Undergraduate Student, Monique Brewer, for her friendship and dedication to this research;

Dr. Carol Trent, Department of Biology, WWU, for her assistance, suggestions, and constructive criticism;

Dr. Spencer Anthony-Cahill, Department of Chemistry, WWU, for his willingness to oversee our efforts in Dr. Prody's absence;

Graduate student Lori Westendorf, for the information and suggestions she provided;

Bernadette Davidson for all of her assistance in my communications with Dr. Prody; and

My loving family, who have never ceased to support and encourage me.

This research was supported in part by a grant from the Bureau for Faculty Research, Western Washington University.

## ABBREVIATIONS

bp	base pairs
CPMV	cowpea mosaic virus
2,4-D	2,4-dichlorophenoxyacetic acid
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	gravity (9.8 m/s <sup>2</sup> )
HIV-1	human immunodeficiency virus 1
mRNA	messenger RNA
MS	Murashige and Skoog
NaOAc	sodium acetate
PCI	phenol:chloroform:isoamyl alcohol
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulfate
sTobRV	satellite of tobacco ringspot virus
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA

TE	Tris EDTA
TobRV	tobacco ringspot virus
Tris	Tris(hydroxymethyl)methylamine
tRNA	transfer RNA
U	units
UV	ultraviolet
VPg	virus-encoded genome-linked protein

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## INTRODUCTION

### **Tobacco Ringspot Virus**

Tobacco ringspot virus (TobRV) infects a variety of monocotyledonous and dicotyledonous plants. Although named after the tobacco plant, TobRV can infect many other types of plants, including both perennial and annual crops. One of the most economically devastating infections caused by this virus is budblight of soybean (Allington, 1946). When infected, buds of the plant become brown and brittle, pods remain underdeveloped, and stems and leaves develop brown streaks. Other plants susceptible to TobRV infection include blueberry, dogwood, spearmint, cherry, and gladiolus (Stace-Smith, 1985).

TobRV is disseminated by nematodes, classifying it as a nepovirus (Stace-Smith, 1985). Nepoviruses are characterized by their nematode transmission vectors and their polyhedral capsids. Embryonic seed tissue can also serve as a viral reservoir; thus infection can spread from seed stocks or planting stocks, or by mechanical inoculation.

Two types of TobRV infection are possible; each type depends on the interaction between the plant and the virus. Some plants are systemic hosts, such as Black Valentine (*Vigna unguiculata*). Systemic infection spreads throughout the plant in an uneven fashion. The manifestations of systemic disease vary with species, age, and other conditions. Although many plants die as a result of such an infection, some merely suffer a reduction in size.

Others, such as cowpea plants, are local lesion hosts. In this case, infection does not spread throughout the plant. Ringspots appear on the leaves of the plants, which can



be necrotic or chlorotic lesions. Necrotic lesions develop as rings with central areas of dead (necrotic) cells. Chlorotic lesions occur when the central cells of a ring-like lesion lose chloroplasts and other pigments. Local lesions result at the site mechanical inoculation with TobRV.

The genome of TobRV consists of two single stranded ribonucleic acid (RNA) molecules, designated RNA-1 and RNA-2. Gel electrophoresis has shown RNA-1 to be approximately 7,800 nucleotides, and RNA-2 is approximately 3,500 nucleotides. As is characteristic of nepoviruses, each of these molecules is separately encapsidated during the viral maturation process. Some capsids may contain two RNA-2 molecules, but none will contain an RNA-1 molecule and an RNA-2 molecule. Both RNA molecules are required for infectivity (Harrison and Barker, 1978). Both molecules are of the (+) sense and have polyadenylated three-prime tails, highlighting their use as messenger RNA (mRNA) in the translation process. A polyprotein is encoded by 7514 nucleotides of RNA-1. Nucleotides 2014 to 583 of RNA-2 encode the viral capsid protein (Buckley, *et al.*, 1993). The capsid polypeptide has a weight of about 13,000 Daltons and forms stable tetramers. Sixty identical tetrameric capsid proteins combine to form the viral capsid.

Also required for infectivity is a five-prime, covalently attached polypeptide. This polypeptide is a virally encoded genome-linked protein (VPg) and is attached to both RNA-1 and RNA-2. Although proteinase treatment of similarly linked cowpea mosaic comovirus (CPMV) does not abolish infectivity, this polypeptide is required for the infectivity of TobRV and tomato blackring nepovirus (TBRV). The VPg is linked at serine 5 to the five-prime uridylylate of each genomic RNA (Zalloua, *et al.*, 1996). VPg presence does not affect *in vitro* translation (Chu *et al.*, 1981).

Viral replication relies on virus-directed RNA polymerase activity. Replicative intermediates have been suggested as a means of RNA proliferation (Rezaian and Francki, 1973). In this model, the (+) sense encapsidated RNAs serve as templates for synthesis of the complementary (-) sense strands. These (-) sense strands then serve as templates for synthesis of the biologically active (+) sense strands.

### **Satellite of TobRV**

Several groups of plant viruses have become associated with small RNA molecules that are not of viral origin. These small RNAs do not have significant sequence homology with genomic viral RNAs and are thus termed “satellites” (Francki, 1985). The first satellite to be identified was that of TobRV. In 1969, Irving Schneider found this new sequence after growth of several generations of TobRV infected greenhouse plants. The satellite of the budblight strain has been cloned and sequenced by Buzayan *et al.* (1986a). It consists of 359 nucleotides.

The (+) sense of satellite of TobRV (sTobRV) is also packaged in a separate viral capsid which is encoded by genomic RNA-2 (Schneider and White, 1976). Up to 20 copies of the satellite can be encapsidated in a single capsid. When the satellite is present, it can account for over 90% of the encapsidated RNA (Schneider and Thompson, 1977). The presence of the satellite results in a dramatic reduction in the degree of viral symptoms (Francki, 1985).

In contrast to the genomic viral RNA-1 and RNA-2, sTobRV lacks the five-prime linked VPg protein and the three-prime polyadenylated tail. At its five-prime end, the satellite has a hydroxyl group and at its three-prime end, a two-prime, three-prime cyclic

phosphate (Buzayan, *et al.*, 1986b). Therefore, the satellite does not show messenger capabilities, and no translation products were found when this sequence was used in a wheat germ translation system (Owens and Schneider, 1977).

The (+) sense of sTobRV is present in circular form in infected tissue (Sogo and Schneider, 1982). Also present in infected tissue are linear multimeric forms of both (+) and (-) sense sTobRV. These multimeric forms consist of tandem repeats of the monomeric, 359-nucleotide form (Keifer *et al.*, 1982). The presence of multimeric forms suggests a rolling circle form of replication. Branch and Robertson (1984) have proposed two possibilities. Both possibilities begin with a circular, unit length (+) sense RNA. In the first model, multimeric (-) strands are made from (+) strand templates. Then, the multimeric (-) strands serve as a templates for a multimeric (+) strands which are cleaved into unit length monomers. In the second model, circular (+) strands are used as templates to generate multimeric (-) strands. These strands are then cleaved into monomeric (-) strands. These (-) strands circularize and act as templates for multimeric (+) strand synthesis. The (+) strands are then cleaved to monomeric length.

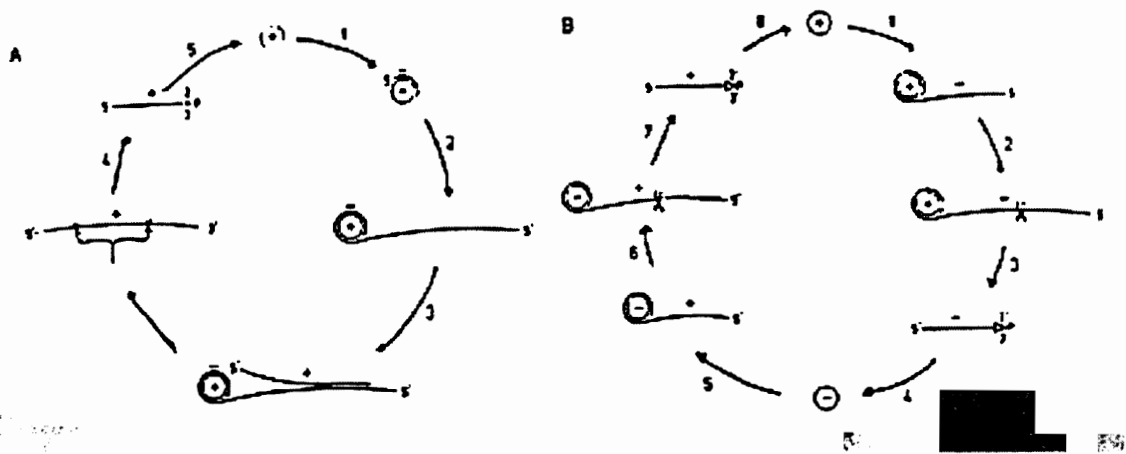


Figure 1. Rolling circle replication. This mechanism of replication was proposed by Branch and Robertson (1984). Model A is favored over B.

The proposal of a rolling circle form of replication is supported by the findings of Prody, *et al.* (1986), who demonstrated that (+) sense multimeric satellite RNAs can undergo a reversible self cleavage reaction. This cleavage generates biologically active (+) sense sTobRV. The cleavage preferentially takes place at a BN\*GUC sequence where B is any nucleotide except A; N is any nucleotide: G, U, and C are guanine, uracil, and cytosine respectively; and \* represents the site of cleavage (Anderson, *et al.*, 1994).

The autocatalytic ability of sTobRV is similar to group I and group II mitochondrial introns, which can excise themselves from preRNA transcripts to form functional mRNA. Group I introns are present in ribosomal RNA (rRNA) genes, mitochondrial rRNA and messenger RNA (mRNA) genes, and in chloroplast transfer RNA (tRNA) genes (Dujon, 1989). These introns rely on a minimal 16-nucleotide sequence for catalytic activity and require specific secondary and tertiary structure for phosphodiester cleavage and ligation (Dinter-Gottlieb, 1986). Beginning with a guanine nucleotide attack of the five-prime splice site, the first exon then attacks the three-prime

splice site releasing the intron. The presence of group II introns has also been shown in mitochondria of higher plants (for review, see Michel, *et al.*, 1989). Similar to group I, group II introns are capable of self-excision but use an adenine-branched lariat intermediate. Such catalytic RNAs have been termed “ribozymes.”

Cleavage of sTobRV is an autocatalytic reaction in which the catalyst is not consumed during the course of the reaction. Hampel and Tritz (1989) have shown that nucleotides 224 to 175 of the (-) sense act as the minimum catalytic region, and nucleotides 53 to 40 are the minimum, 14-nucleotide substrate. The catalytic site does not fold in the form of a hammerhead, as seen in (+) sense sTobRV (Foster and Symons, 1987). Instead, the (-) sense sTobRV folds into a six-helix hairpin ribozyme (Figure 2). Ligation is favored over cleavage for the hairpin structure, whereas the hammerhead structure derived from (+) sense sTobRV favors cleavage (Fedor, 1999). The ligation propensity of the (-) sense strand affirms its candidacy as a rolling circle replicative intermediate.

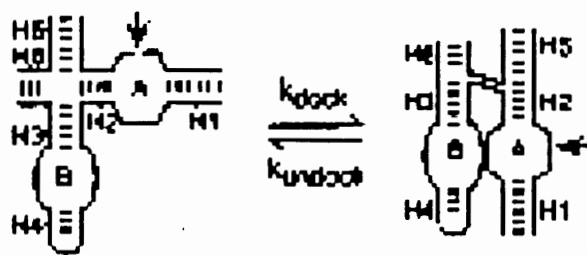


Figure 2. Hairpin ribozyme. Shown in the docked conformation on the right and the undocked conformation on the left. Biological activity results from the docked conformation. The arrow points to the cleavage and ligation site. From Fedor, 1999.

The ability of sTobRV to attenuate the symptoms of tobacco ringspot virus infection in susceptible plants and its ability to act as a ribozyme have led to a suggested mechanism of viral attenuation. It is proposed that sTobRV has the ability to cleave RNA-1 and RNA-2 into truncated, non-biologically active RNAs. This assertion is supported by the proven ability of sTobRV to cleave other viral RNAs. For instance, it has been shown to cleave human immunodeficiency virus 1 (HIV-1), hepatitis B virus, hepatitis C virus, and human papillomavirus (Lian *et al.*, 1999).

### **Plant Cell Culture Suspension**

The use of a plant cell culture allows for the study of sequence origin without the possible complication of contamination. The culture is prepared and grown in aseptic conditions to prevent any bacterial or viral contamination. A selective herbicide that acts as an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), is used to induce undifferentiated cell division. The cell culture can serve as a source of mitochondrial DNA from uninfected cells.

### **Statement of Purpose**

This study has been conducted in an attempt to elucidate the origin of the sTobRV sequence. The work of Florian E. Christensen has served as the impetus for this study. Through cellular fractionation and sTobRV (-) <sup>32</sup>P probing, she demonstrated the possible presence of the sTobRV sequence in the DNA of mitochondria from tobacco cells (Christensen, 1991). It has been our aim to replicate and verify these results.

## MATERIALS AND METHODS

### Maintenance of a cell culture

Cells of *Nicotiana tabacum* L. cv. BY-2 were maintained in culture (Kikkawa *et al.*, 1982). This rapidly growing cell line produced immature chloroplasts. Cells were cultured every four days in a Murashige and Skoog media (MS) (Murashige and Skoog, 1962). MS media was modified to include 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 180 mg/L KH<sub>2</sub>PO<sub>4</sub>, 100 mg/L myo-inositol, 1 mg/L thiamine-hydrochloride, and 30 g/L sucrose, adjusted to pH 5.7 with 10 mM KOH (Bolton *et al.*, 1986). Stock 2,4-D solution was made by dissolving 0.05 g of 2,4D in 5 mL absolute methanol. Slowly, 50 mL of warm deionized water were added; then the solution was brought to 500 mL with the addition of deionized water.

Cells were grown in 250 mL Erlenmeyer flasks at room temperature with constant rotary shaking at 170 rpm and low intensity fluorescent lighting. Flasks were plugged with cotton wrapped with gauze and covered with foil. Cotton plugs were reused, and foil was replaced before autoclaving. Cells were cultured with 15 mL of four-day growth culture and 35 mL of media.

After approximately 10 rounds of subculturing, a drop of culture was spotted on a glass slide and viewed and 1000X magnification using a Nikon light microscope to assess for the possibility of bacterial contamination.

All subculturing, media transfer, and slide spotting were performed in a laminar flow hood. Seventy-five percent ethanol was used to sterilize the screen and bench.

Non-disposable glassware was autoclaved, and media was purified by vacuum filtration through a 0.2  $\mu\text{m}$  filter (Nalgene).

### **Isolation of mitochondrial DNA**

Mitochondrial DNA was isolated from BY-2 cell culture using a protocol modified from Li, *et al.* (1996). Cells were harvested four days after subculturing. The total contents of each flask (50 mL) were emptied into 50 mL plastic conical vials with screw cap closure. Vials were centrifuged in a clinical centrifuge for 10 minutes to pellet cells (approximately 600 x gravity (g)). The supernatant was discarded.

Homogenization buffer (50 mM Tris, 25 mM ethylenediaminetetraacetic acid (EDTA), and 1.25 M NaCl, pH 8) was prepared by the addition of beta-mercaptoethanol to 10 mM and egg albumin (Sigma) to 0.1%. Buffer was chilled for 20 minutes at  $-20^{\circ}\text{C}$ .

Cells were combined with homogenization buffer in a 1:1 (v/v) ratio. This suspension (40-45 mL) was added to a Pyrex homogenization tube. Homogenization was carried out by plunging with a tightly fitting Teflon plunger for 10 minutes. The homogenate was distributed into 27 mL ultracentrifuge tubes (Beckman). Samples were centrifuged at 1,500 x g for 10 minutes in a Sorvall SA-600 rotor. Supernatant was drawn off and collected in a 250 mL graduated cylinder.

Mitochondria were lysed by the addition of sodium dodecyl sulfate (SDS) to 0.5% (w/v), N-Lauroylsarkosine to 2% (w/v), and proteinase-K to 50  $\mu\text{g}/\text{mL}$ . The solution was mixed by briefly stirring. The sample was then distributed into 50 mL plastic conical vials and incubated in a  $37^{\circ}\text{C}$  water bath for 75 minutes.



A phenol chloroform isoamyl alcohol (PCI) extraction was prepared. Phenol (containing 1 mg/mL hydroxyquinoline), chloroform, and isoamyl alcohol were then added to each tube in a 25:24:1 (v/v) ratio, respectively. Samples were centrifuged in a clinical centrifuge for 3 minutes (approximately 650 x g). Aqueous layers were collected and extracted using the same method. Aqueous layers were drawn off and an equal volume of chloroform was added. Tubes were centrifuged in a clinical centrifuge for 3 minutes (approximately 650 x g).

Aqueous layers were drawn off and aliquotted into ultracentrifuge tubes. Two volumes of ice cold ethanol and 1/10 volume of sodium acetate (NaOAc) were added to each tube. Tubes were placed on ice for 30 minutes. Tubes were balanced on a top loading balance and centrifuged for 15 minutes at 10,000 x g in a Sorvall centrifuge using an SA-600 rotor. The supernatant was drawn off and tubes were set upside down to dry on a Kim Wipe. Pellets were resuspended in 1 mL Tris EDTA, pH 8 (TE) and stored at -20° C.

RNA was removed from DNA samples by ribonuclease (RNase) treatment. Seven  $\mu\text{L}$  RNase solution (1 unit/ $\mu\text{L}$ ) were added to 100  $\mu\text{L}$  of sample. This reaction was incubated in a 37° C water bath for 30 minutes. Samples were stored at -20° C.

## Virus isolation

A solution containing 250 to 275 mL of 0.02 M NaKHPO<sub>4</sub> (12 mM Na<sub>2</sub>HPO<sub>4</sub>, 8 mM KH<sub>2</sub>PO<sub>4</sub>) 0.1 mM EDTA, pH 7 was mixed in a blender. Three mL of 50% K<sub>2</sub>HPO<sub>4</sub> (w/v), 1.25 g ascorbic acid, and 250 µL beta-mercaptoethanol were added. With slow blending, 100 to 125 g of frozen, tobacco ringspot infected *Vigna unguiculata* cv. Blackeye 5 leaf tissue were added. An equal volume of chloroform-butanol 1:1 (v/v) was added. The mixture was centrifuged in a Sorvall GS-A rotor for 15 minutes at 13,180 x g. Then, 0.2 M NaCl and 4 µL beta-mercaptoethanol per mL solution were added to the supernatant. Over a 30 minute span, polyethylene glycol (PEG) was added to 8% (w/v). PEG was dissolved with stirring for 30 minutes. Mixture was chilled overnight at 4° C.

Samples were centrifuged at 2,600 x g for 30 minutes in a GS-A rotor. Pellets were resuspended in 100 mL 0.2 M NaCl, 8% (w/v) PEG, 0.1 mM EDTA, 0.02 M phosphate, and 0.5 µL/mL beta-mercaptoethanol and allowed to sit for 70 minutes. Samples were centrifuged at 4° C for 30 minutes in a GS-A rotor at 13,180 x g. Pellets were resuspended in approximately 0.02 M NaKHPO<sub>4</sub> and allowed to sit for 60 minutes. Samples were centrifuged in an SA-600 rotor for 10 minutes at 14,470 x g. Aqueous layers were transferred to 27 mL ultracentrifuge tubes (Beckman). Tubes were balanced on an analytical balance and centrifuged in a Beckman ultracentrifuge Ti-60 rotor for 2 hours at 236,925 x g. The supernatant was decanted, and pellets were dried and covered with 500 µL 0.2 mM EDTA, pH 6.5. Samples were chilled overnight at 4° C.

Samples were centrifuged for 1 minute at 7,000 rpm. The supernatant was stored in 50% (v/v) glycerol.

## **Viral RNA extraction**

Eppendorf tubes were filled with approximately 100  $\mu$ L of virus solution. An equal volume of 2X extraction buffer was added (100 mM Tris-HCl, 4 mM EDTA, pH 8.0, 1% (w/v) SDS, 0.16 mg/mL bentonite). Samples were heated in a 37° C water bath for 20 minutes. An equal volume of phenol with 1 mg/mL hydroxyquinoline was added. Chloroform and isoamyl alcohol were added to each tube to make a 25:24:1 ratio of phenol:chloroform:isoamyl alcohol. Tubes were vortexed for 20 seconds and centrifuged at 4° C for 5 minutes at approximately 6,500 x g. Using the supernatant, an ethanol precipitation was conducted with 2.7 X volume of 95% ethanol and 1/10 volume 3.3 M NaOAc. Samples were mixed by micropipeting and placed in a dry ice ethanol bath for 15 minutes. Samples were centrifuged at 4° C for 15 minutes at approximately 13,000 x g. The supernatant was removed and pellets were washed with cold 70% ethanol and centrifuged as above for 10 minutes. The supernatant was drawn off and tubes were dried upside down on a Kim Wipe.

Pellets were resuspended in 450  $\mu$ L diethyl pyrocarbonate (DEPC) water. DEPC was prepared with the addition of 0.1% (v/v) DEPC to deionized water. The solution was shaken for 12 hours at 37° C and then autoclaved. Another ethanol precipitation was conducted using 1/10 volume 3 M NaOAc, pH 5.2, and 2 volumes ice cold absolute ethanol. Samples were placed in a dry ice ethanol bath for 15 minutes and then centrifuged at 4° C, approximately 12,000 x g. The supernatant was drawn off and pellets were dissolved in 20  $\mu$ L DEPC water.

RNA yield was analyzed by electrophoresis on a 0.8% (w/v) agarose gel in 1X TBE (diluted from a 5X stock of 0.045 M Tris borate and 0.001 M EDTA). Ethidium

bromide was added to 0.5 µg/mL. Gels were viewed on a 312 nm ultraviolet light (Fisher Biotech) and photographed with a Polaroid camera (Fisher Biotech).

### **Polymerase chain reaction**

Polymerase chain reactions (PCRs) were conducted in a 30 µL volume using Deep Vent DNA Polymerase (New England Biolabs). For each reaction, 12 µL 10X reaction buffer, 2.4 µL 100 mM MgSO<sub>4</sub>, 12 µL 10X dNTPs, and 4 µL of each primer were used. Templates and sterile deionized water were added to 30 µL. Two units of Deep Vent DNA Polymerase were added to each reaction just before placing in thermal cycling machine.

Primers were designed from the known sequence of the budblight strain of TobRV (Figure 4) using the Primer 3 program offered by the Michigan Institute of Technology to select the sequences and Williamstone Primer Design program to assess the secondary structure possibilities. In the first set of reactions, 18 nucleotide primers were used. The forward primer annealed to the five-prime sequence of the satellite and was designated sTobRVL1-5, and the reverse primer annealed to the three-prime end of the satellite sequence and was designated sTobRVRev1-359 (Figure 4).

Primers used in the second set of reactions were longer. The forward primer, sTobRVF61, is 24 nucleotides and began at nucleotide 61 of the satellite sequence. The reverse primer, sTobRVR353, was 25 nucleotides and began, from the three-prime end, at nucleotide 353 (Figure 3).

5'

ACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGGACTGTC

AGGTGGCCGAAAGCCACCACGTAAACTAGTGAACCCTGCTGCGTAGCGTAGG

GGTCTGCTACCTCGTTGGAGGTGGAGATTGTAGCCTTCGTGTGGGCGCGGCG

GTGTAGCTAGTCAAGGCGTACCAGGTAATATACCAACAACGTGTGTTTCTCTG

GTTGACTTCTCTGTTTGTGTGTCATTGGTTCCCGGATCTCGCATTAGCGGC

GACGGGGTATTCTCATTCGACATGGAAGTTTGAGAGACCGCGCCTCTACACT

ATGCGCGGCCGGGGCGAATCCAAATTGTTCTAGCCCGATACCCTGTC

3'

Figure 3. sTobRV sequence of budblight strain with PCR primers. The satellite sequence shown with arrows beneath the sequence to represent primers used in the first set of reactions. Arrow heads point in the direction of complementary strand synthesis.

The sequence of sTobRV has been inserted into a pSP65 (Promega) vector and termed T8. As a template in PCR reactions, T8 provides a size and specificity standard.

A positive control reaction was used for each PCR reaction. The template was drosophila DNA. The primer sequences were derived from conserved regions of eukaryotic 18S rRNA corresponding to the five-prime end of the 18S rRNA gene (NS1 and NS2) (Cano, *et al.*, 1993). The positive control reaction was mixed as follows: 3  $\mu$ L 10X reaction buffer, 100 mM MgSO<sub>4</sub>, 10X dNTPs, 15 pmol of each primer, and 150 ng of template. The primers and template were a gift from Dr. Carol Trent, Western Washington University.

Reactions were run in a Robocycler™ thermal cycling machine. Samples were incubated at 95° C for 3 minutes, then proceeded through 30 cycles of 95° C for 1 minute, 45° C for 1 minute, and 72° C for 1 minute.

Reactions were analyzed on a 1% (w/v) agarose gel in 1X TBE. Five  $\mu\text{L}$  of each reaction were loaded on the gels. Gels were electrophoresed for approximately 2 hours at 71 volts, stained with ethidium bromide at 0.5  $\mu\text{g}/\text{mL}$ , and visualized on a 312 nm ultraviolet lamp box (Fisher Biotech).

### **Sequencing**

Samples were electrophoresed on a 1% (w/v) agarose gel in 1X modified TAE (diluted from 50X stock of 40mM Tris acetate, 0.1 mM EDTA, pH 8.0). Gels were stained with 0.5  $\mu\text{g}/\text{mL}$  ethidium bromide and visualized with a 312 nm ultraviolet light (Fisher Biotech). Bands were cut from gels using a disposable scalpel. Excised bands were centrifuged at 5,000 x g for 15 minutes at 4° C in gel nebulizing Eppendorf tubes (Millipore), which allow passage of nucleic acids and gel buffer to the bottom of the tube and retain agarose in a disposable filter. Tubes were then centrifuged for 5 minutes at 12,000 x g at room temperature.

Samples were purified using a PCR purification kit (Quaigen). The water storage option was used.

According to the Thetagen sequencing protocol, 40 ng of PCR product were used for fragments of 100 – 200 base pairs, and 125 ng were used for fragments of 200 – 500 base pairs. The appropriate masses were pipetted into individual Eppendorf tubes. One primer was added to each Eppendorf to a total of 4 pmol. Tubes were then opened and covered with parafilm. The parafilm was pierced with a sterile pipette tip. Samples were then vacuum centrifuged with heating (Speedyvac) for 30 to 60 minutes until dry. Tubes were placed in a 50 mL conical vial and mailed to Thetagen in a padded envelope.

Sequencing results were viewed using Chromas® personal computer program.

Analysis of sequence homology with known sequence was conducted using MacVector

3.5 Sequence Analysis Program (IBI, 1991).

## RESULTS

### **Maintenance of BY-2 cell culture**

BY-2 cells were grown in a rotary shaker and grew rapidly producing bright yellow cells, hence the acronym “BY” (Kikkawa *et al.*, 1982). This color was used as an indicator of the exponential phase of growth; cells degraded to a brownish solution within a few weeks of subculturing. Cells grown in a plywood case with fluorescent lighting on three sides (constructed at WWU) were more prolific than those grown in a bench top shaker which was illuminated only by fluorescent room lighting and sunlight during daytime hours.

### **Isolation of mitochondrial DNA**

BY-2 cells sedimented quickly with centrifugation in a clinical centrifuge but also sedimented well upon standing. Just a few cell aggregates remained in the supernatant fraction after centrifugation. PCI extractions were most evenly distributed when each component (phenol with hydroxyquinoline, chloroform, and isoamyl alcohol) was added separately to each tube. Use of a master mix would allow for aqueous – organic separation prior to use of the PCI mixture.

When the cells were growing well, 250 mL of 4-day-old cultures were harvested. Using this volume of thick, healthy cell culture resulted in a very large, smeared pellet after ethanol precipitation and centrifugation. Extraction of mitochondrial DNA from these cultures led to the isolation of 2.4 mL of approximately 10 ng/ $\mu$ L DNA. The DNA was analyzed by electrophoresis on an ethidium bromide stained agarose gel. Incubation



with RNase resulted in less streaking in each lane of the gel, indicating that the DNA was in the high molecular weight band, not in the low molecular weight streaking (Figure 4).

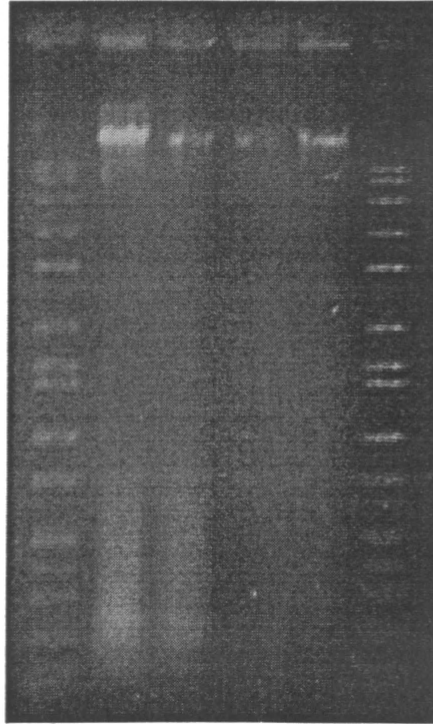


Figure 4. Ribonuclease treated mitochondrial DNA. Lanes two and three show isolated nucleic acids prior to ribonuclease treatment. Lanes four and five show the same samples after ribonuclease treatment. Outside lanes are HiLo DNA marker.

Isolated DNA was digested with various restriction enzymes. The most complete cleavage was seen with *Sau3 AI* restriction enzyme (Figure 5), because it recognizes the four base sequence \*GATC in the five-prime to three-prime direction, where \* is the site of cleavage. A four base sequence would be more common than a longer recognition sequence. Less complete cleavage occurred with all other enzymes used. *EcoR I* produced the next most complete digestion; its recognition sequence is, from five-prime to three-prime, G\*AATT, where \* is the site of cleavage.

Table 1. Restriction enzymes used in the digestion of mitochondrial DNA and their respective recognition sites. Sequences are shown five-prime to three-prime and cleavage sites are denoted with an \*.

Restriction Enzyme	Recognition Site
Sau3A I	*GATC
EcoR I	G*AATTC
Pst I	CTGCA*G
Hind III	A*AGCTT
BamH I	G*GATCC
Bgl II	A*GATCT
Vsp I	AT*TAAT
Turbo NAE I	GCC*GGC

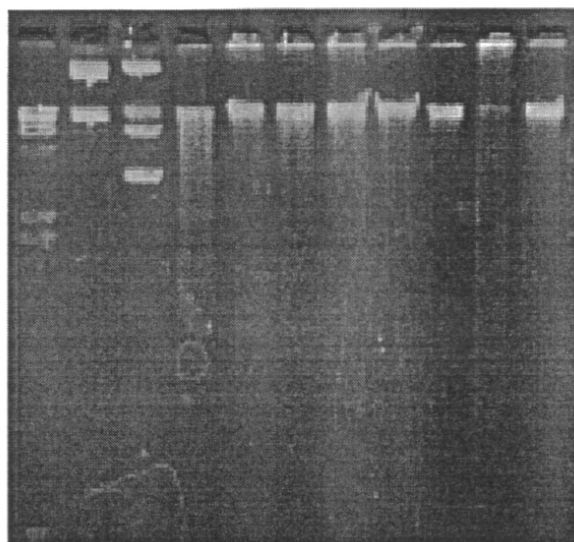


Figure 5. Restricted mitochondrial DNA. Lanes two and three show undigested and BamH I digested T8 plasmid respectively. Lanes four through 11 show mitochondrial DNA digests with EcoR I, Pst I, Hind III, BamH I, Bgl II, Vsp I, Sau3A I, and Turbo NAE I respectively. Lane I is  $\lambda$  Hind III digested DNA as a marker.

### **Virus isolation and viral RNA extraction**

TobRV was isolated from frozen leaf tissue. Chloroform-butanol, beta-mercaptoethanol, and PEG were used separate out plant tissue. Approximately 100 g of frozen leaf tissue yielded 2 mL of virus solution. Virus solution was stored in 50% glycerol.

RNA extraction from virus was carried out from solutions stored in glycerol. Samples were diluted with the addition of five volumes of 2X extraction buffer. Initial isolations were carried out on the bench top, and did not include the use of DEPC water. Most of the RNA from these preparations was degraded; only the satellite was discernable on the gel (Figure 6), so an RNase free approach was taken. Bench tops and surrounding shelves were wiped down with RNase AWAY (Molecular Bio-Products), all glassware and gel boxes were wiped down with DEPC water, and all solutions were made with DEPC water. Again, complete RNA degradation was observed.

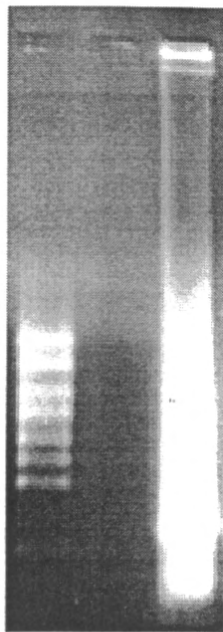


Figure 6. Viral RNA extraction. The right lane shows degraded TobRV and sTobRV RNA. The band represents sTobRV RNA. The left lane is an RNA ladder.

### **Polymerase chain reaction**

PCRs were performed on restriction enzyme digested mitochondrial DNA as the template. Initially, PCRs used primers that were 18 base pairs in length. These primers were designed to anneal to the five-prime and three-prime ends of the sequence of the budblight strain of sTobRV. A 30 cycle reaction using Sau3A I digested mitochondrial DNA yielded two products as determined by agarose gel electrophoresis. These products were approximately 350 base pairs and 150 base pairs respectively. These bands were cut from the gel for sequencing. There was a fair amount of background smearing on the gel as well (Figure 7).

The T8 plasmid, containing the sequence of the budblight strain of sTobRV, was used as a positive control in all PCR reactions. It was restricted with BamH I, which linearizes the plasmid.

This reaction was repeated and yielded six bands with less smearing. Still, a band at approximately 350 base pairs was stronger than the rest (Figure 8). Multiple bands in the product, however, suggested that the primers were not specific.

At this point, it was also realized that Sau3A I cleaves within the sTobRV sequence, so Pst I digested mitochondrial DNA was used as the template in future PCRs.

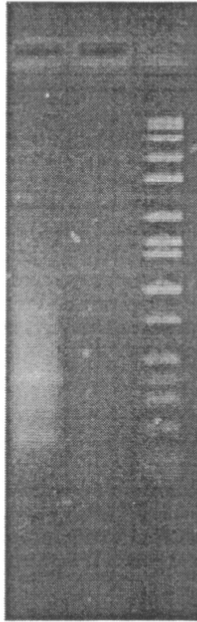


Figure 7. PCR product using original primers. Bands at ~350 bp and ~150 bp represent PCR products from 18 bp primers. The right lane is HiLo DNA marker.

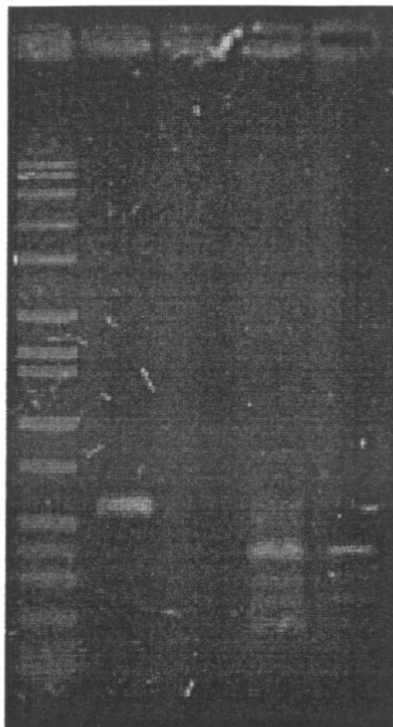


Figure 8. Duplication of PCR using original primers. Lanes four and five represent PCR products obtained from 18 bp primers. Lanes two and three are positive and negative controls respectively. Lane one contains HiLo DNA marker.

New primers were designed to increase specificity. The forward primer anneals approximately one quarter of the way into the satellite sequence from the five-prime end, and the reverse primer anneals at the three-prime end. These primers are longer than those used at first; they are 24 and 25 base pairs long. A PCR reaction with these primers and Pst I digested mitochondrial DNA as template yielded a smeary product. One band was slightly brighter than the rest at approximately 300 base pairs, the expected size (Figure 9).

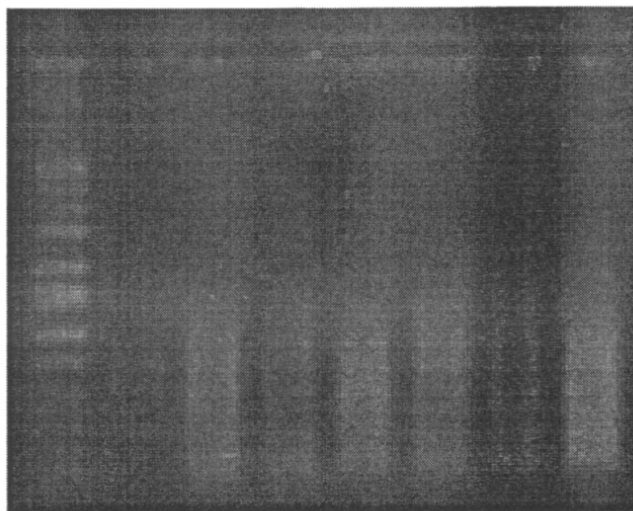


Figure 9. PCR product using redesigned primers. Primers were 24 and 25 bp long. Lanes two through eight represent a gradient of annealing temperatures increasing from 44° C to 56° C.

### **Sequencing**

Products from the first PCR were prepared for commercial sequencing by Thetagen. The ~350 base pair product was sent separately from the ~150 base pair product. Each sequence was read in one direction by the forward primer and in the other direction by the reverse primer. Unread nucleotides were replaced in the reverse primed sequences by comparison to the forward primed sequence; the same was possible for

forward primed sequences by using the reverse primed sequences. Good, discernable results were produced; there were only 3 unreadable nucleotides in the ~350 base pair fragment and no unreadable nucleotides in the ~150 base pair fragment. Sequences were analyzed using MacVector 3.5 Sequence Analysis Program (IBI, 1991), and neither showed homology with the budblight strain of sTobRV.

## DISCUSSION

In search of the origin of the sequence of sTobRV, this research has focused on PCR amplification of isolated mitochondrial DNA. The mechanism of viral symptom attenuation was also investigated. Because it causes budblight of soybean, tobacco ringspot virus has economic and agricultural significance. The ability of the satellite of tobacco ringspot virus to attenuate viral symptoms makes this a relevant and potentially beneficial study.

### Mitochondrial DNA

Following the work of Florian Christensen (1991), this investigation has focused on the mitochondrial DNA from uninfected tobacco cells. During the isolation, other cellular components are discarded and the mitochondrial fraction is saved. Because of the difference in density of chloroplasts and mitochondria, the chloroplasts are left behind when the cellular homogenate is centrifuged at 1,500 x g; the supernatant solution is used and the pellet is discarded. Therefore, the nucleic acids remaining in the sample are of mitochondrial origin and findings pertain directly to the mitochondria of *Nicotiana tabacum* L. cv. BY-2 cells.

These tobacco cells, which are maintained in a sterile culture, remain uninfected by any virus. Therefore, no nucleic acids of viral origin are present in these cells nor in the nucleic acids isolated from these cells.



## **Polymerase chain reaction**

Because the sequence of the budblight strain of sTobRV is known, primers can be specifically designed within that sequence. Specific primers allow for the use of PCR as a tool to probe the sequence of the mitochondrial DNA. The size of the mitochondrial genome makes it a diverse template. The possibility of primers annealing to similar – but not identical – sequences is increased with such a large template. Longer primers are more specific, because they require a longer complimentary sequence, which is less likely to be similar to another sequence.

Specificity is also increased by using nested primers. The PCR product of a reaction with satellite-specific primers is used as the template in another PCR using a different forward primer and the same reverse primer. Therefore, the anticipated product of the first PCR should contain an internal sequence that is homologous with sTobRV. Then, primers that are internal and specific to sTobRV will anneal to the template. This technique can demonstrate the presence of desired sequence elements within the original PCR product (Figure 10).

5'

ACCGGATGTGCTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGGACTGTC  
 AGGTGGCCGAAAGCCACCACGTAAACTAGTGAACCGTGCTGCGTAGCGTAGG  
 GGTCTGCTACCTCGTTGGAGGTGGAGATTGTAGCCTTCGTGTGGGCGCGGCG  
 GTGTAGCTAGTCAAGGCGTACCAGGTAATATAACCACAACGTGTGTTTCTCTG  
 GTTGACTTCTCTGTTTGTGTGTCATTGGTTCCCGGATCTCGCATTAGCGGC  
 GACGGGGTATTCTCATTTCGACATGGAAGTTTGAGAGACCGCGCCTCTACACT  
 ATGCGCGGCCGGGGCGAATCCAAATTGTTCTAGCCCGATAACCCTGTC

←-----→  
 ←-----→

3'

Figure 10. Nested primers for the sTobRV sequence. Shown is the sequence of the budblight strain of sTobRV. The nested forward primer is shown with a dashed line. The same reverse primer is used in the nested priming reaction.

### Mitochondrial DNA restriction

Although the mitochondrial genome of *Nicotiana tabacum* L. cv. BY-2 has not yet been sequenced, mitochondrial genomes of other plants have been shown to have genomes as large as 186,000 bp to 366,000 bp. The mitochondrial genome of *Merchantia polymorpha* is 186609 bp (Oda, *et al.*, 1992) and that of *Arabidopsis thaliana* is 366,924 bp (Unsel, *et al.*, 1996). Because mitochondrial genomes are relatively large, enzyme restriction was used to make the template into smaller, more easily denatured segments. Although Sau3A I produced the most completely digested sample, restriction with Sau3A I was perhaps a poor choice. Its four-base cleavage recognition site is found within the sequence of the budblight strain of sTobRV. Therefore, cleavage of mitochondrial DNA with Sau3A I can cleave the satellite sequence. Because of the relatively complete digestion produced by this restriction enzyme, it is likely that the satellite sequence, if present, was cleaved. Therefore, it is logical that the sequenced PCR product was not homologous with the sTobRV sequence: that template would have been cleaved by the Sau3A I digest.

### **Mitochondrial introns and satellite replication**

Because the satellite of tobacco ringspot virus is a small, autocatalytic RNA, it is suggestive of a pre-cellular world (Diener, 1989). In this hypothesis, RNAs were free-living and responsible for their own replication. The satellite sequence may be a remnant of this pre-cellular life.

However, there is a striking similarity between the action of the sTobRV and that of mitochondrial introns. Consistent with our search for the sTobRV sequence, introns are also found in organelles, such as mitochondria. Lambowitz (1989) has speculated that RNA molecules like sTobRV have evolved from these intron-type elements. Group I introns are capable of self-excision and circularization. Experimental evidence suggests that group I introns have the ability to insert into predetermined locations in intronless genes (Volker Knoop, 1994).

Mitochondria of higher plants have also been shown to contain group II introns. Group II introns are also capable of self-splicing (for review, see Michel, *et al.*, 1989). The satellite of TobRV is found in circular forms in infected cells (Keifer, *et al.*, 1982). Self excision could provide a mechanism for the origin of this sequence.

This hypothesis is also consistent with the proposed rolling circle mode of replication. Excised, circularized satellite RNAs would fit easily into the hypothesized role of circular template (Branch and Robertson, 1984). After (+) sense preRNA transcription, the satellite could excise itself and a (-) sense circular template could be generated for satellite replication.

Because of the ability of the satellite to circularize, and the proposed ability of group I introns to insert into intronless genes, it is possible that the sequence of sTobRV is permuted in the mitochondrial DNA. This possibility makes adequate primer design difficult, because it is impossible to tell if primers are in areas of complete, uncleaved sequence or if they are oriented toward each other. Because of these complications, a Southern blot may be more effective in determining presence of the satellite sequence. A labeled probe of (-) sense sTobRV will be hybridized with blotted mitochondrial DNA and RNA.

#### **Satellite cleavage of viral RNA**

The satellite sequence of tobacco ringspot virus has been proposed to cleave the viral RNA-1 and RNA-2. This proposal is validated by the evidence that sTobRV can cleave other viral RNAs, such as the human immunodeficiency virus (Hampel, 1998). In order to assess the cleavage of viral RNAs by sTobRV, viral RNA was isolated from infected plants and run out on a gel (Figure 6, p.20). However, only large smears of degraded RNA were obtained even in an RNase free environment. Possibly, the presence of the satellite with viral RNA-1 and RNA-2 is causing a cleavage reaction during the isolation procedure.

#### **The origin of satellite RNA**

By using more specific PCR primers, differently digested mitochondrial DNA, and Southern blots, the presence of the sTobRV sequence in the mitochondrial DNA of uninfected *Nicotiana tabacum* L. cv. BY-2 cells will be assessed.

Identification of the sTobRV sequence within the mitochondrial DNA could lead to further studies of its multiplicity and its capability to act as a transposable intron. Since sTobRV has been shown to attenuate viral symptoms, identification of the origin of this sequence may eventually provide agricultural and health benefits.

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