INDUCTION OF VIRAL RESISTANCE IN PLANTS BY dsRNA INTERFERENCE

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE GRADUATE SCHOOL OF THE

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COLLEGE OF ARTS AND SCIENCE

BY

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TEXAS WOMAN'S UNIVERSITY
DENTON, TEXAS

11/04/05
Date

To the Dean of the Graduate School:

I am submitting herewith a dissertation written by Thomas W. De Lany entitled "Induction of viral resistance in plants by dsRNA interference". I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Molecular Biology.

John E. Knesek
Dr. John E. Knesek, Major Professor

We have read this dissertation
and recommend its acceptance.

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Accepted by:

Jennifer Martin
Dean of the Graduate School

DEDICATIONS

This is dedicated to my wife, Kim. Without her as my partner, source of strength and inspiration, I would not have completed this work.

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ABSTRACT

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INDUCTION OF VIRAL RESISTANCE IN
PLANTS BY dsRNA INTERFERENCE

DECEMBER 2005

Double-stranded RNA (dsRNA) has been shown to promote interference of gene expression of endogenous genes that contain homologous sequences. This interference is referred to as post-transcriptional gene silencing or PTGS. PTGS appears to be a nucleotide sequence specific defense mechanism that can target both endogenous and exogenous messenger RNA (mRNA). PTGS employs a yet unexplained RNA degradation mechanism.

In this research, *in vitro* synthesized dsRNA homologous to two different segments of the genome of the bean common mosaic necrosis virus (BCMNV) was applied to bean plants to determine if the dsRNA affected the susceptibility of the plant to BCMNV infection. The results indicated that the application of dsRNA reduced the number of local lesions per leaf and reduced plant death by approximately 20%. Using dsRNA that expressed homology to different regions of BCMNV did not result in any difference in the ability of dsRNA to inhibit viral replication. Within the parameters of concentration of dsRNA used, no difference in the effectiveness of viral inhibition of the dsRNA was observed. A relationship was observed between the time interval between the

application of dsRNA and viral local lesions. The greater reduction of local lesions was observed on leaves that had the greater time interval between the application of the dsRNA and viral inoculation. Systemic viral resistance was observed when the primary leaf was treated with dsRNA and trifoliate leaf was inoculated with the virus. No viral resistance was observed in plants that developed from seeds produced by dsRNA treated plants that survived viral inoculation.

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LIST OF ABBREVIATIONS

BCMV: bean common mosaic virus

BCMNV: bean common mosaic necrosis virus

cDNA: complementary DNA

CHS: chalcone synthase gene

DEPC: diethylpyrocarbonate

DNA: deoxyribonucleic acid

dsRNA: double stranded RNA

EDTA: ethylene diaminetetraacetate

ELISA: enzyme-linked immunosorbent assay

MCS: multi-cloning site

mRNA: messenger RNA

ORF: open reading frame

PMMoV: pepper mild mottle virus

PTGS: post-transcripional gene silencing

RNA: ribonucleic acid

RNAi: RNA interference

siRNA: short interfering RNA

VPg: viral protein genome linked

CHAPTER I

INTRODUCTION

Double-stranded RNA (dsRNA) has been shown to promote interference of plant gene expression if the dsRNA exhibits homology to the plant gene. In 1990 in an attempt to improve flower color, Napoli et al. over expressed the chalcone synthase (CHS) gene via a transgene in petunia. Instead of getting enhanced flower color, the absence (suppression) of color in the flowers was observed (23). Attempts to explain the absence of flower color resulted in the discovery that messenger RNA (mRNA) for CHS was not present in the cytoplasm of plants that exhibited suppressed flower color. Further examination of the process of transcription revealed that while mRNA was not present in the cytoplasm both pre-mRNA and mRNA were present in the nucleus (12). Since RNA molecules encoded by both the transgene and the homologous endogenous gene were not present in the cytoplasm, the phenomenon was termed co-suppression or post-transcriptional gene silencing (PTGS) (23, 29). RNA silencing has since been discovered in the fungus *Neurospora crassa* (26) and termed quelling. It was then discovered in the nematode *Caenorhabditis elegans* (22) and called RNA interference (RNAi).

Without mRNA there can be no protein synthesis in the cell. Protein synthesis is a fundamental process of cellular activity, since proteins perform many important functions in an organism. They support the organism, transport other substances, regulate and coordinate metabolic and bodily activities, respond to chemical and

mechanical stimuli, protect against disease and perform enzymatic activities. The instruction for protein synthesis resides in deoxyribonucleic acid (DNA), in unique nucleotide sequences known as genes. Ribonucleic acid serves as the link between genes and their products.

While the mechanism for PTGS is not completely understood, it is thought that it results from the specific degradation of homologous RNA (12, 31). While the process greatly reduces host or viral mRNA by degrading it in the cytoplasm, it does not interfere with transcription (8, 21). Short interfering RNA (siRNA) molecules are present only in transgenic plants that display or exhibit PTGS. In transgenic plants that do not display PTGS, these siRNA's are not present (13). These small dsRNA molecules appear to be an integral part of PTGS in many other organisms as well (6, 15, 20 and 30). Observation of plants in nature reveals that PTGS may be a natural defense against viral infection (2, 25). Plants may have a system that detects dsRNA from replicating viruses (31), which is the signal for plants to initiate an RNA-mediated anti-viral defense (26). Messenger RNA is degraded only in the presence of siRNA and only within the sequence spanned by the dsRNA (32). Since PTGS is observed in different species and all employ genes exhibiting homology, it may be that PTGS is an ancient defense mechanism.

In 2001 there were roughly 80 billion U. S. dollars in the international trade of bean seeds. Of the approximately 2400 pathogens that attack dry beans, one third is represented by viruses. Bean common mosaic virus (BCMV) and bean common mosaic necrosis virus (BCMNV) cause the most widely distributed disease in the various cultivars of the common bean plant (4).

Different cultivars of the common bean plant exhibit temperature sensitive resistance to BCMV. This resistance to BCMV is due to the presence of an *I* (inhibitor) gene in the bean cultivars (1, 19). In bean varieties that lack the *I* gene, the bean common mosaic necrosis virus (BCMNV) induces common mosaic symptoms similar to those caused by BCMV. However, in the cultivars that possess the dominant *I* gene, BCMNV induces a lethal hypersensitive reaction (18). Shortly after viral infection, necrotic symptoms develop on either the primary or trifoliate leaves. The virus moves from infected cells to the secondary and primary veins surrounding them, which causes the veins to become brown-black; the virus and necrosis spreads into the phloem tissue. As the virus moves rapidly through the vascular tissue, wilting and then necrosis occurs in the stem, roots, new leaves and meristem tissue. Plant death soon follows.

To distinguish between the two viruses, tests such as enzyme-linked immunosorbent assay (ELISA) can be performed or the two viruses can be differentiated by their reaction on plants containing the *I* gene. Cultivars that contain the *I* gene are resistant to BCMV, but highly susceptible to BCMNV.

BCMNV is a member of the *Potyviridae* family. Members of this family are flexuous rods between 650 and 900 nm in length and 11 to 15 nm in diameter. The genome, which varies between 8.5 and 10 kilobase pairs (kbp), is a plus sense (+) single stranded RNA (ssRNA) with a VPg (viral protein genome linked) covalently bound to its 5' end and a 3' poly A tail (28). The genome consists of a single open reading frame (ORF), and is expressed as a single polyprotein that is co- and/or post-translationally

cleaved into either intermediates or functional viral proteins (Table 1) by three viral encoded proteinases (13, 19, and 28) (Figure 1).

BCMNV is a seed borne pathogen that is also transmitted non-persistently by aphids (13). Bean plants infected with the virus develop severe vascular necrosis in the leaves, stems and roots; the viral infection is frequently referred to as black root disease (9). The genome of BMCNV is 9612 nt in length. BCMNV was once considered a Type A serovar of BCMV, specifically the NL-3 strain.

Our hypothesis was PTGS might provide an effective treatment for the control of BCMNV infection. If dsRNA were able to induce resistance to BCMNV infection in bean plants, what conditions would have the greatest effect or influence on the inhibition of viral replication? Variables such as concentration of dsRNA, time of application of dsRNA prior to challenging the plant with BCMNV and the site of homology of the dsRNA to the genome of the BCMNV were examined. Since this virus appears to move through the plant systemically (5), bean plants were tested to determine if dsRNA moved through the plant systemically as well. Seeds from plants that exhibited PTGS were tested to determine if PTGS might be a seed borne phenomenon. Plants that exhibited PTGS were assayed to determine if siRNAs were present.

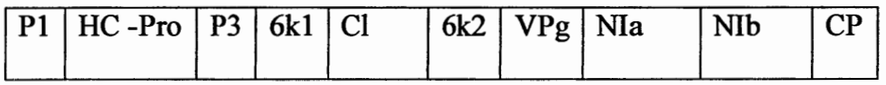


Figure 1. Representative *Potyviridea* polyprotein. Cleavage site of functional protein are indicated by vertical lines. Abbreviations of the names of the viral proteins are indicated within the ORF. (29)

Table 1. Proteins encoded by the Potyvirus genome (29)

Protein	Function
P1	protease, autocleavage, RNA binding, symptomology (?)
HC	Pro-protease, aphid transmission, genome amplification, suppression of gene silencing
P3	polyprotein processing, symptomology(?)
6K1	symptomology, silencing
CI	RNA helicase, cell-to-cell movement
6K2	attachement to membranes, hydrophobic region
VPg	viral protein genome linked, viral replication
NIa	cysteine peptidase, majority of cleavage activities.
NIb	RNA dependent RNA polymerase
CP	cell to cell and systemic (long distance movement), virus assembly.

CHAPTER II

MATERIALS AND METHODS

Materials

Commercial Kits

The following commercial kits were used in this research: Gene Amp XL PCR Kit (Perkin Elmer); SuperScript First Strand Synthesis for RT-PCR (Invitrogen); Elu-Quik DNA Purification (Schleicher & Schuel); SequiTherm EXCEL™ II DNA Sequencing Kit (Epicentre Technologies); FastLink Ligation and Screening Kit (Epicentre Technologies); Wizard® Miniprep; Qiagen Plasmid Midi Kit (Qiagen); QIprep® Spin Miniprep (Qiagen); Protect™ Bacterial Preservers (Key Scientific Products); RETROscript™ First Strand Synthesis Kit (Ambion); mirVana™ miRNA Isolation Kit (Ambion)and MAXscript (Ambion).

Restriction Enzymes

The following restriction enzymes were purchased from either New England Biolabs or Invitrogen (GibcoBRL): *Eco*RV, *Xba*I, *Hin*dIII, *Pst*I and *Xho*I.

Bacteria

Escherichia coli DH10B (F⁻ *mcrA* Δ*(mrr-hsd*RMS-mcrBC) φ80d*lac*ZΔM15 Δ*lac*X74 *deo*R*rec*A1 *end*A1 *ara*D139 Δ*(ara, leu)*7697 *gal*U *gal*K λ⁻ *rps*L *nup*G) was used to make competent cells, which served as recipients of recombinant plasmids containing the

required DNA templates. Bacterial cells were stored at -80°C using Protect™ Bacterial Preservers (Key Scientific Products).

Media

Medium used for bacterial growth was either NZYM or LB (Qbiogene, Inc.) and was solidified with Bacto Agar (DIFCO) at 1.6 g/100 ml. Transformed cells were grown on NZYM medium with kanamycin (50 μg/ml) and IPTG (1 mM). NZYM or LB broth was used to grow selected transformants for purification of plasmid DNA.

Buffers, Primers, Reagents and Chemicals

The following reagents were used: IPTG; kanamycin; 5X TBE Liquid Concentrate (Amresco); Long Ranger Gel Solution (Cambrex Bio System); ammonium persulfate (Amresco); SeaKem® GTG^{R} Agarose (Cambrex Bio System); agarose (Sigma); kanamycin sulfate (Amersco); ethidium bromide; 0.02 M K_2PO_4 (pH 7.5); 2 mM EDTA (pH 8.0); absolute ethanol; 70 % ethanol; primers (BioSynthesis) and UTP ($^{32}\alpha$P) (PerkinElmer™).

Laboratory Equipment

The following equipment was used: G24 Environmental Incubation Shaker (New Brunswick Scientific Co. Inc.); Environmental Control Room (Lab-Line Instruments, Inc.); Dry Type Bacteriological Incubator (Blue M Electric Company); Electrophoresis Documentation and Analysis System (EDAS) 120 (Kodak); GeneAmp PCR System 2400

(Applied Biosystems); Spectrofuge 16M (Labnet); Controlled Environmental Chamber (Sherer); Beckman JS-HS (Beckman) and rotors JA14 and JS 7.5; Barnstead EASYPure UF (Barnstead); Hybridization Incubation Model 310 (Robbins Scientific) and DNA sequencer Model 4000L (LI-COR),

Computer Software

The following computer software or programs were used: Gene Construction Kit [Textco, Inc. (a SciQuest Company)]; Kodak DS ID (Kodak) and Vector NTI™ Suite (InforMax, Inc.).

Methods

Design and construction of recombinant plasmids used as templates for dsRNA synthesis

Purified BCMNV (Washington isolate) was available in our laboratory. The Washington isolate is identical to the Michigan isolate, except for 15 additional nucleotides that encode five amino acids in the NIa (nuclear inclusion a) protein. The nucleotide sequence of the Michigan isolate was used to design primers. Dr. Nathan Mills' laboratory (Texas Woman's University) transcribed the purified RNA of the BCMNV into cDNA and then into dsDNA.

Two pairs of recombinant plasmids were constructed. One pair served as the DNA template for one of the dsRNA molecules used in the various experiments while the second pair served as the template for the second dsRNA molecules used. One set of primers was designed so the DNA template would synthesize a dsRNA molecule that expressed homology to a nucleotide sequence near the 3' end of the BCMNV genome.

The nucleotide sequence for the forward primers was: 5' TTT TTT CTC Agg gCg TAT CAA CTC ACA TAC 3'. The nucleotide sequence for the reverse primers was: 5' TTT TTT CTC TCA ggA AAT CCT gCT ATC gC 3'.

The DNA was amplified using the above primers and Gene Amp XL PCR kit (PerkinElmer) by a PCR (25 cycles: denature, 94° C for 1 min; anneal, 55° C for 1:30 min and elongation, 72° C for 5 min). The PCR mixture contained the following: 3.3XL buffer, 15 µl; forward and reverse primer, 1 µl each (25 pmole/µl); $Mg(OAc)_2$, 2.2 µl; rTth polymerase, (2U/µl; DNA template, 3 µl (30 to 60 ng/µl) and H_2O, 22.8 µl. The PCR product was purified by Elu-Quik DNA Purification kit and the DNA was analyzed by electrophoresis on an agarose gel (0.8%).

The vector used to construct the recombinant plasmids was pZErO™-2.1 (3.3 kb plasmid) and was digested with the restriction enzyme *Eco*RV, resulting in blunt end dsDNA. Digestion was confirmed by comparing digested and undigested pZErO™-2.1 by electrophoresis on a 0.8% agarose gel. Digested pZErO™-2.1 and the newly amplified 3' dsDNA underwent blunt end ligation using an Epicenter Technologies Fast Link Ligation and Screening kit. Since the ligation was blunt end, two different orientations of the dsDNA were obtained. Competent *E. coli* DH10B bacterial cells were transformed with the recombinant plasmid and plated on NZYM medium that contained IPTG (1 mM) and kanamycin (50 µg/ml). Only bacterial cells that contained a recombinant plasmid grew since pZErO™-2.1 contains a kanamycin resistant gene. Additionally, pZErO™-2.1 contains a lethal gene, *ccd*B, which is fused to the C-terminal of *lacZ*. This gene is inducible by IPTG. The MCS (multicloning site) is within the *lacZ* open reading frame

(ORF). Insertion of the DNA fragment into the MCS disrupted the expression of *lacZ-ccdB* fused gene permitting the growth of recombinants. Orientation of the dsDNA in the recombinant was determined by isolating the plasmids of possible transformants using either a Wizard® Plus Miniprep or QIprep® Spin Miniprep kit. The recombinant plasmids were approximately 4.6 kbp. The recombinant plasmid was analyzed using the restriction enzyme *Xba*I. There were 2 *Xba*I restriction sites; one was within the MCS and the other was near one end of the dsDNA insert. In one orientation of the DNA insert, the two restriction sites were only 55 nucleotides apart and digestion with the restriction enzyme *Xba*I resulted in a 4.6 kbp fragment. In the other orientation, the two restriction sites were about 1.4 kbp apart. Digestion with *Xba*I resulted in 2 fragments, one approximately 3.2 kbp and the other 1.4 kbp. To further verify the orientation of the DNA insert, DNA sequencing was done using an Epicentre Technologies SequiTherm EXCEL™ II DNA Sequencing Kit with M13 forward and M13 reverse primers (LI-COR) and LI-COR 4000L Automated DNA Sequencer. One orientation was used to synthesize the sense strand of the RNA molecule while the other orientation was used to synthesize the antisense strand of the RNA molecule.

The second set of recombinant plasmids was constructed from a recombinant plasmid (designated 2bgl) available in our laboratory (7) that was used to sequence the BCMNV genome of the Washington isolate. The transcription of this DNA template yielded an RNA molecule that expressed homology to a nucleotide sequence near the 5' end of BCMNV. The plasmid containing the desired viral DNA sequence was extracted and purified using Qiagen Plasmid Maxi Prep. The DNA was digested with two

restriction enzymes, *Eco*RV and *Hin*d III. An Epicentre Technologies End-It™ DNA REPAIR kit was used to fill the 5' overhang, followed by blunt end ligation utilizing the Epicentre Technologies Fast Link DNA Ligation & Screening kit. The ligation resulted in recombinant plasmids in both orientation of dsDNA in relation to the SP6 promoter site. Competent *E. coli* DH10B cells were transformed with the plasmid. Selection for the transformants, which were kanamycin resistant, was done on NZYM agar plates containing the antibiotic kanamycin. Isolation of the plasmid DNA of the transformant was done using either Wizard® Plus Miniprep or QIprep® Spin Miniprep kit. The orientation of the DNA with respect to the SP6 promoter of the original recombinant plasmid was known. Digestion of this plasmid DNA with the restriction enzymes *Pst*I and *Xho*I resulted in a large fragment of approximately 4 kbp. Digestion with the same restriction enzymes of a recombinant plasmid with the DNA insert in the opposite orientation resulted in 2 fragments, one about 750 bp and the other 3.3 kbp. One orientation was used to synthesize the sense strand of the RNA molecule while the other orientation was used to synthesize the antisense strand of the RNA molecule. To further verify the orientation of the recombinants, DNA sequencing using an Epicentre Technologies SequiTherm EXCEL™ II DNA Sequencing Kit was done.

Competent cells

E. coli DH10B bacteria were grown in 43 ml of NZYM or LB medium in a 250 ml baffled DeLong flask at 37°C in an incubator shaker to an optical density of 0.6 (A_{600nm}). Cell suspensions were centrifuged in a Beckman J2-HS at 3500 rpm in a JS-7.5

rotor at 0°C for 5 min. The supernatant was discarded and the pellet was suspended in 25 ml of cold calcium rubidium (CaRb) solution (0.01 M RbCl, 0.1 M CaCl) and incubated in an ice water bath for 30 min. The cells were centrifuged at 2500 rpm for 5 min in a JS-7.5 rotor at 0°C. The supernatant was discarded and the pellet was resuspended in 2 ml of cold CaRb solution and stored on ice until ready for use.

Storage of desired transformants

The desired bacterial transformants with the recombinant plasmids in the appropriate orientations were stored on beads at –80°C. These beads were plated to NZYM-kanamycin agar plates as needed.

Viral Specific Synthesis of dsRNA

The virus specific DNA was transcribed into dsRNA using an Epicentre AmpliScribe™ SP6 High Yield Transcription kit. *E. coli* bacteria containing the required recombinant plasmid were grown on NZYM-kanamycin agar plates. Isolated bacterial colonies were harvested and the plasmid DNA was extracted as outlined above. The plasmid DNA was linearized with either the restriction enzymes, either *Xba*I or *Not*I. This resulted in the DNA template downstream from the nearby SP6 promoter site. The DNA was quantified by spectrophotometer. The final concentration of template DNA used in dsRNA synthesis was between 60 and 100 ng/µl. The dsRNA was transcribed using the following: linearized DNA template (each orientation), 1 to 3 µl; 10X SP6 buffer, 2 µl; 100 mM ATP, UTP, CTP and GTP, 1 µl each; 100 mM DDT, 2 µl and

AmpliScribe SP6 enzyme, 2 µl; RNase free water was added to bring the total volume to 20 µl. The mixture was incubated at 37°C for a minimum of 2 hr to a maximum of 4 hr. Since both templates were present in the mixture, the nascent transcribed ssRNAs annealed.

The dsRNA was concentrated and purified by ethanol precipitation using the following: dsRNA, 20 µl; 5 M NaCl, 4 µl; 0.2 mM EDTA, 3.4 µl and 2.5 volume of absolute ethanol (67 µl). The mixture was incubated overnight at -20°C. A dsRNA pellet was obtained by spinning the mixture in a microcentrifuge at maximum speed (14,000 rpm) for 15 min. The supernatant was removed by aspiration and the dsRNA pellet was washed in 70% ethanol (20 µl). The supernatant was removed by aspiration. The dsRNA pellet was resuspended in ultra pure water (100 µl). This product was incubated with RNase A and T_1 (3 µl RNase: 300 µl RNase digestion buffer) at 37°C for 30 min and the mixture was activated by adding 225 µl of inactivation buffer and incubating at -20°C for 1 hr or more. This product was filtered on an NICK Column to remove free nucleotides. The dsRNA was quantified by spectrophotometer using an extinction coefficient of 1 O.D. unit equal to 50 µg/ml at 260 nm. Formation of dsRNA was confirmed by agarose gel electrophoresis as outlined above. The dsRNA was stored at -20 °C.

Plant Material

The common bean plants (cultivars Top Crop, Sutter Pink and Advance Pinto) were grown in a growth chamber with 16 hr light at 850 to1000 foot-candles and 8 hr dark at 25 ° to 30 °C. Top Crop plants contained an *I* gene (inhibition gene) and were

therefore highly susceptible to BCMNV. BCMNV induces a lethal hypersensitive reaction in Top Crop plants. Sutter Pink and Advance Pinto do not contain an *I* gene and do not die if infected with BCMNV. Monthly planting of either Sutter Pink or Advance Pinto and inoculating the new plants with virus from a virally infected plant maintained fresh BCMNV.

Isolation of virus

Leaves (0.5 g) from infected plants were triturated and mixed with 2 ml of 0.02 M K_2PO_4 buffer. The mixture was spun for 2 min at 14,000 rpm in a microcentrifuge to separate the cell debris from the cell lysate, which contained the viral particles. The cell lysate was diluted in order to achieve a gradient in ten percent increments (i.e. the 80% viral concentration contained 80 µl of the original cell lysate and 20 µl of 0.02 M K_2PO_4 buffer).

Application of dsRNA and virus to plants

Top Crop plants were challenged with different concentrations of either 5' or 3' dsRNA and/or virus. Double stranded RNA and/or virus were applied mechanically to the primary leaves of the plants. Leaf size averaged 4096 mm^2. Virus was extracted from either infected Sutter Pink or Advanced Pinto plants. One-half gram of an infected leaf was triturated and then 2 ml of 0.02 M K_2PO_4 was added to the pulverized leaf. The mixture was spun in a microcentrifuge for 2 min at maximum rpm (14,000) to separate the virus from the leaf debris. Application of dsRNA and/or virus was accomplished in a

half leaf assay using a mixture of the abrasive carborundum (600 mesh 0.5 g) and ultrapure H_2O (5 ml). Textured rubber gloves were worn to add to the abrasiveness. Application consisted of placing by micropipette 15 µl of the carborundum mixture in the middle of the leaf. The leaf was supported by stiff cardboard. One swipe was made with the forefinger upward, downward and to each side of the application site. The appropriate concentration of dsRNA and/or virus was applied in the same manner to leaves that had received the carborundum mixture. Positive controls were plants that received only virus (15 µl/half leaf) and negative control plants received only 15 µl of 0.02 M K_2PO_4

Three to five plants were used within each sample group, resulting in 12 to 20 half leaf samples. Analysis of effectiveness of dsRNA on viral inhibition was based on two criteria: plant survival and/or reduction in lesions. Viral lesions usually appeared 48 hr after viral challenge but were counted 72 hr after viral challenge. The numbers of viral lesions were averaged for each concentration sample.

Isolation of siRNA

To determine if siRNA was present in plants that exhibited PTGS, a trifoliate leaf from one these plants was picked. The petiole and major veins were removed and the remainder of the leaf was cut into small squares of approximately 0.5 cm. A *mir*Vana™ miRNA Isolation kit (Ambion) was used to extract and purify any siRNA present. The elution process yielded 100 µl. The procedure was applied to three other leaf types: primary leaf from a plant that exhibited PTGS, a primary and trifoliate leaf from a virally infected plant.

The siRNA, which is double stranded, was denatured using formamide. Two microliters of the eluted product from each sample was incubated with 10 µl of formamide, 2 µl of 10X MOPS buffer (3-[N-morpholino] propane sulfuric acid) and 4 µl of formaldehyde at 55°C for one hr. Electrophoresis of the samples occurred in a 10X MOPS-deionized formaldehyde 3% agarose gel. The gel was pre-run at 5 V/cm for 5 min. Samples were loaded into the wells and the gel was run at 5 V/cm for about 3 hr. A Northern blot using a neutral transfer buffer system was performed and the membrane was exposed to ultraviolet light (254 nm) @ 120 J/cm in order to cross-link the RNA.

Two RNA probes were constructed using the DNA of recombinant plasmids that had served as the source for the dsRNA applied to the plants. The probes were synthesized with radioactive $^{32}\alpha$P uridine triphosphate. Specific activity for the probes was 8.5 X 10^6 to1.17 X 10^7 cpm/µg of RNA.

The membrane was pre-hybridized with 10 ml of Ultrahyb for 1 hr at 42° C. Four microliters of the probe (1.17 X 10^7 cpm) was added to the hybridization buffer and the membrane was hybridized overnight. The membrane was washed with 30 ml of 2X saline-sodium citrate (SSC) and 0.1 % of sodium dodecylsulfate (SDS) three times, 50 ml of 2X SSC and 0.1% SDS for 5 min.

Data analysis

To analyze the lesion data, one way ANOVA were was performed. The null hypothesis, that the difference is not significantly different (i.e. due to chance) is rejected if P is small. A confidence level of 95% was used. If the P value of 0.01 or less resulted,

the null hypothesis was rejected; the difference was not due to chance and was therefore statistically significant.

Since this virus is 100% fatal to Top Crop plants, inhibition of virus was measured by plant survival. One of the hallmarks of PTGS is the presence of siRNA. Plants that survived viral challenge were selected at random and cell lysates were assayed for small (21-25 nt) RNA utilizing techniques outlined above.

CHAPTER III

RESULTS

The purpose of this research was to determine whether dsRNA homologous to different regions of BCMNV would induce resistance to BCMNV infection in the common bean plant when the plant received an application of dsRNA prior to viral challenge. Two different segments of dsRNA, which were homologous to the genome of the BCMNV, were used. One segment corresponded to a nucleotide sequence near the 5' end, nucleotides 1940 to 2697, of BCMNV and was designated 5'dsRNA; the other segment corresponded to a nucleotide sequence near the 3'end, nucleotides 4881 to 6248, and was designated 3'dsRNA. The 5' dsRNA segment was approximately 757 nucleotides in length and the 3'dsRNA segment was 1367 nucleotides in length.

Construction of recombinant plasmids for production of dsRNA

Recombinant plasmids were constructed in order to have a ready supply of DNA, which served as a template for the synthesis of RNA molecules used in this research. Two pairs of recombinant plasmids were constructed, one for the 5' dsRNA and the other for the 3' dsRNA fragment. One member of each pair contained the DNA that served as the coding strand for the sense RNA molecule while the other member contained the DNA in opposite orientation; this served as the coding strand for the anti-sense RNA molecule.

Digestion of the DNA fragment, which served as the template for the sense and antisense strands of 3' dsRNA, with the restriction enzyme *XbaI*, resulted in two different orientations of the insert. In one orientation the two restrictions sites were only 55 bp apart; in the other orientation the two restriction sites were approximately 1.4 kbp apart. In the orientation where the restrictions sites were only 55 bp apart, only one DNA fragment approximately 4.6 kbps was observed on an agarose gel (Figure 2, lane 11). In the orientation where the restrictions sites were approximately 1.4 kbp apart, two DNA fragments were observed on an agarose gel, one approximately 1.4 kbp and the other 3.3 kbp in length (Figure 3, lane 7).

A recombinant plasmid, which was used to sequence the genome of the Washington isolate of BCMNV (7), was available in our laboratory. This transformant contained the DNA that served as the template for the 5' dsRNA and the orientation of the viral DNA was known. Incubation of the DNA from the possible recombinant plasmids resulted in two different fragments, which were identifiable on an agarose gel. The orientation of the original recombinant plasmid DNA was known; incubation of this DNA fragment with the restriction enzymes resulted in a large fragment of approximately 4 kbp on an agarose gel as the two restriction sites were about 10 nucleotides apart (Figure 4, lanes 2, 3 and 4). The DNA insert in the opposite orientation resulted in two fragments after incubation with the restriction enzymes, one approximately 800 bp and the other 3.3 kbp (Figure 4, lane 5 and 7). Orientation of the recombinant plasmid DNA was confirmed by DNA sequencing.

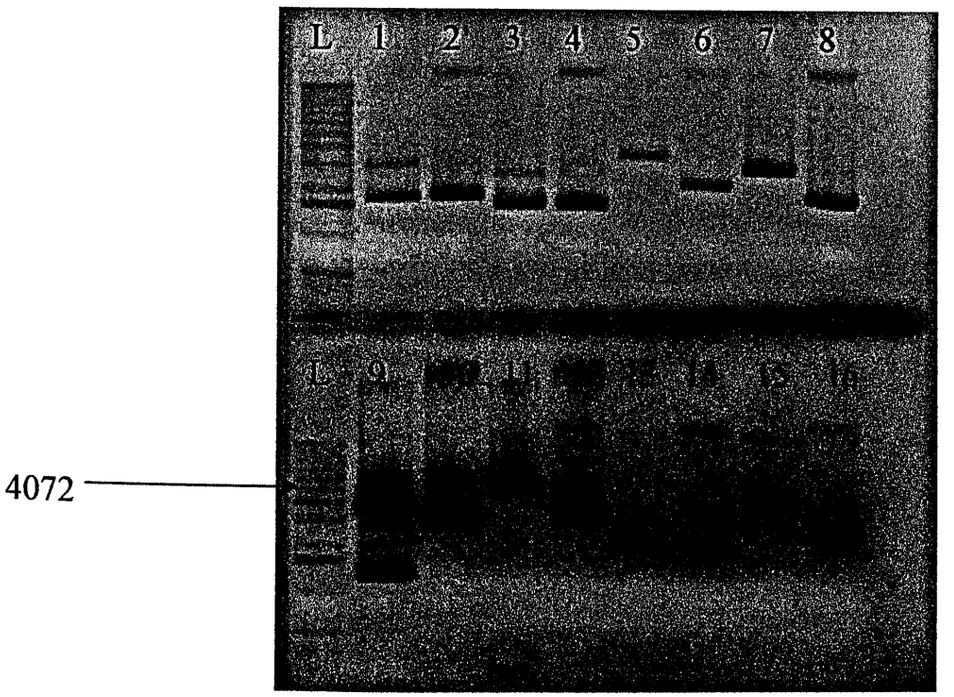


Figure 2. BCMNV 3' recombinant plasmid DNA samples. Samples were incubated with *Xba*I (odd number lanes) and without (even number lanes). Samples were analyzed on a 0.8% agarose gel (11.1V/cm for ~ 30 min). Lane L, 1 kb ladder.

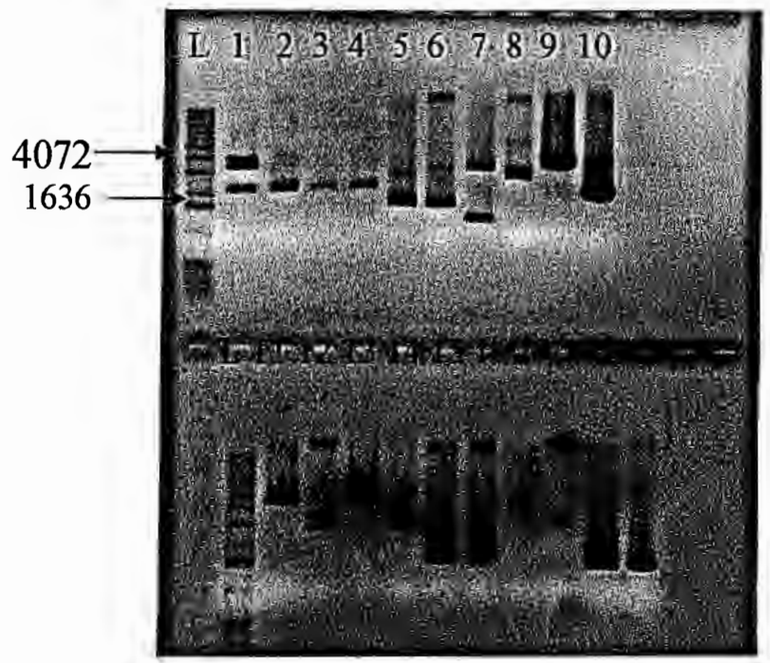


Figure 3. BCMNV 3' recombinant plasmids DNA samples. Samples were incubated with *Xba*I (odd number lanes) and without (even number lanes). Samples were analyzed on a 0.8% agarose gel (11.1 V/cm for ~ 30 min). Lane l, 1 kb ladder.

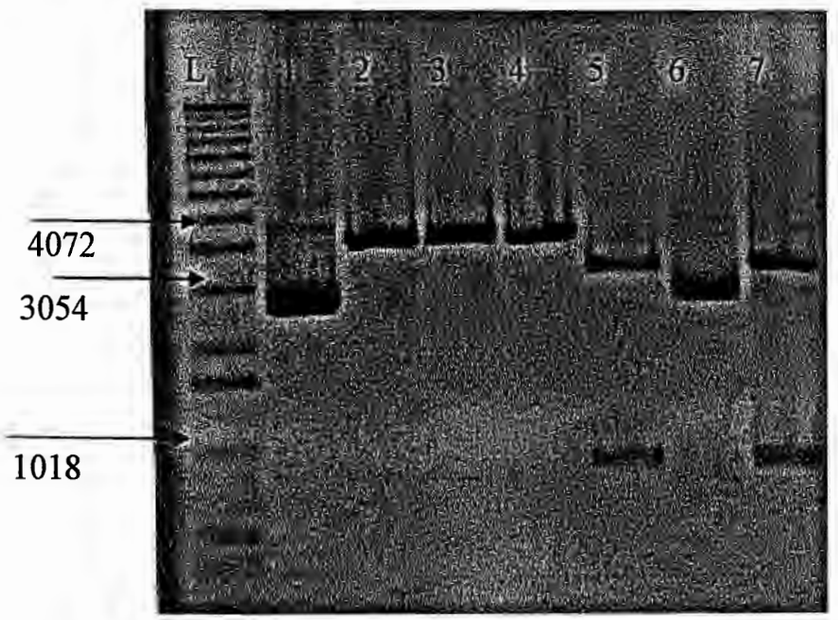


Figure 4. BCMNV 5' recombinant plasmids DNA samples. Samples were incubated with the restriction enzymes *Xho*I and *Pst*I (odd lanes and without (even lanes). Plasmid DNA in lane 3 was incubated with *Pst*I only; plasmid DNA in lane 4 was incubated with *Xho*I only. Samples were run on 0.8% agarose gel (9V/cm for apprx. 1 hr).

Effect of the viral concentration on inhibition of virus

A 10 percent serial dilution of virus was made. The virus was then applied to primary leaves and a linear relationship was observed between the dilutions of virus used and the number of viral lesions (Figure 5). Viral infectivity decreased with dilution.

Effect of different concentration of dsRNA on inhibition of virus

Different concentrations of dsRNA were applied to the leaves of Top Crop plants 24 hr prior to viral challenge to ascertain the effect of concentration of dsRNA on viral inhibition. The various concentrations of 3'dsRNA showed significant reduction in the number of viral lesions (Figure 6). Similar results were obtained using 5' dsRNA (Figure 7). Statistical analysis of the data between the number of viral lesions on plants that received dsRNA prior to viral challenge and the number of viral lesions on plants that received virus only was made by one way ANONA (Table 2). The results indicated that an application of dsRNA to plants 24 hr prior to inoculation with BCMNV significantly reduced the number of local lesions; however, within the parameters of concentration used, there was no statistical difference in reduction of lesions between concentration groups. The degree of confidence was 95%.

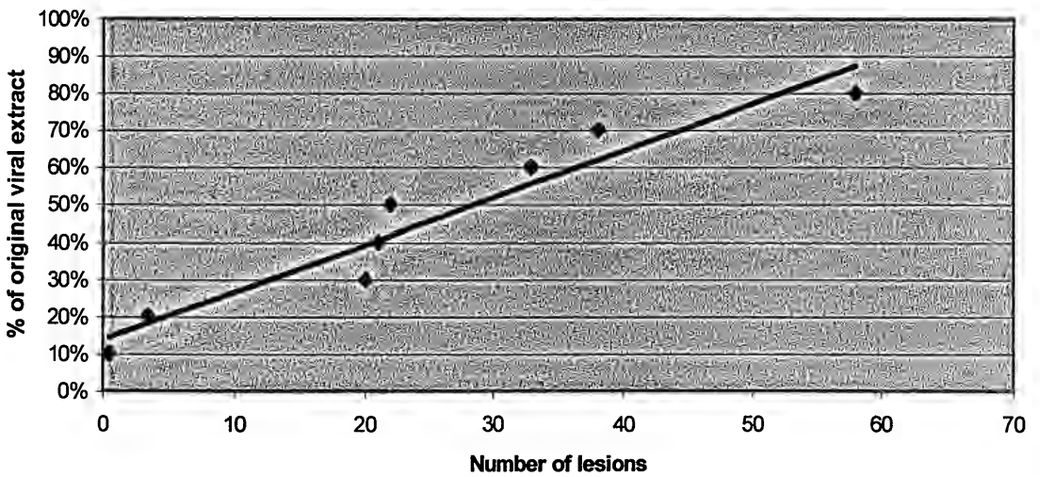


Figure 5. Comparison of viral dilutions to the number of local lesions. Viral extract was prepared by triturating 0.5 g of infected leaves with 2 ml of 0.02 M K_2PO_4 buffer. The cell lysate was diluted in order to achieve a gradient in ten percent increments (i.e. the 80% viral concentration contained 80 µl of the original cell lysate and 20 µl of 0.02 M K_2PO_4 buffer). The various concentrations of the original viral sample were applied to primary leaves in a half leaf assay and the number of lesions counted per half leaf.

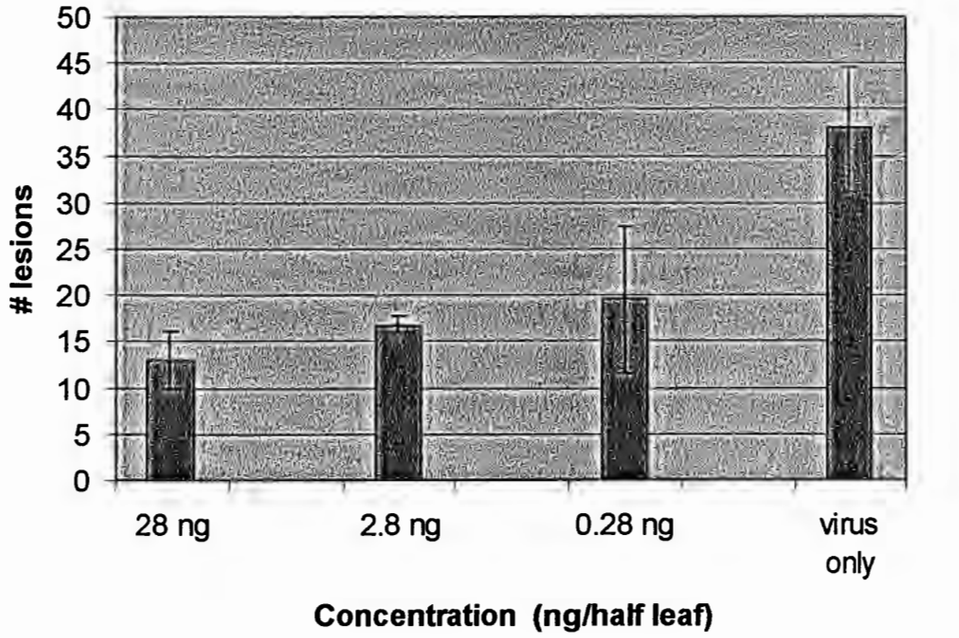


Figure 6. Concentration comparison of 3'dsRNA. Different concentrations of 3' dsRNA were applied to primary leaves in a half leaf assay and plants were challenged with virus 24 hr after the application of dsRNA. Lesions were counted 72 hr after viral challenge and comparison between concentration groups was made.

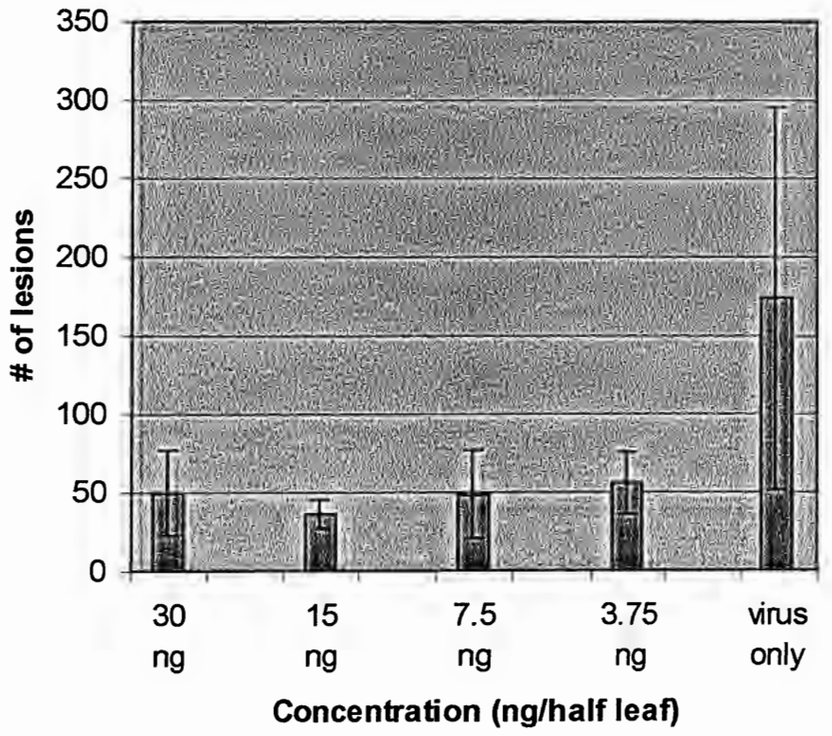


Figure 7. Concentration comparison of 5' dsRNA. 5'dsRNA was applied to primary leaves in a half leaf assay and the plant was challenged with virus 24 hr after the application of dsRNA. Lesions were counted 72 hr after viral challenge and comparison between concentrations were made.

Table 2. Concentration comparison using dsRNA.
The number of viral lesions on plants that received different concentrations prior to viral challenge compared to the number of lesions on plants that received virus only

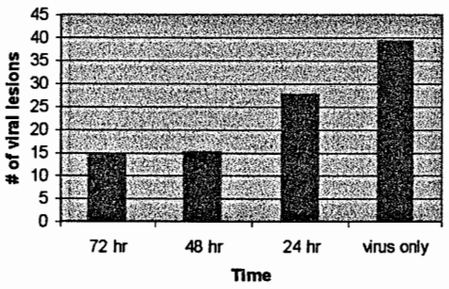
Treatment	Avg. no. of viral lesions per half leaf	% Reduction in viral lesions	P value
3' dsRNA			
28 ng/half leaf	13	66	<0.01
2.8 ng/half leaf	16.81	57	<0.01
0.28 ng/half leaf	19.58	50	<0.01
virus only	39		
28 ng compared to 2. 8 ng			ns
28 ng compared to 0.28 ng			ns
2.8 ng compared to 0.28 ng			ns
5' dsRNA			
30 ng/half leaf	47.75	72.5	<0.01
15 ng/half leaf	36.25	79	<0.01
7.5 ng/half leaf	49	72	<0.01
3.75 ng/half leaf	55	68	<0.01
virus only	173		
30 ng compared to 3. 75 ng			ns
30 ng compared to 15 ng			ns
30 ng compared 7.5 ng			ns
15 ng compared to 3.75 ng			ns
15 ng compared to 7.5 ng			ns
7.5 ng compared to 3.75 ng			ns

ns = nonnsignificant

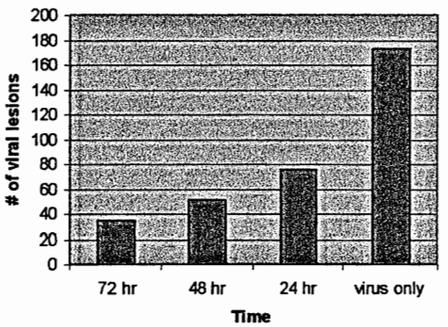
Effect of time of application of dsRNA on inhibition of virus

To determine the effect of time of application of dsRNA on viral inhibition, dsRNA molecules of similar molarity were used. The concentration of the 3'dsRNA was 4 ng/µl and the concentration of the 5'dsRNA was 2 ng/µl. Since the 3'dsRNA molecules contained nearly twice as many nucleotides as the 5'dsRNA molecule, the molarity of the two samples was similar. Fifteen microliters of either 5' or 3' dsRNA was applied mechanically to Top Crop plants in a half leaf application. One group of plants received the dsRNA 72 hr prior to viral challenge, another group received dsRNA 48 hr prior to viral challenge and the third group received the dsRNA 24 hr prior to viral challenge. Lesions were counted 72 hr after viral challenge and the average number of viral lesions per group was obtained (Figure 8). The number of viral lesions on the plants that received dsRNA prior to viral challenge was compared to the number of lesions on plants that were inoculated with virus only using one way ANOVA (Table 3) and the results indicted that the time of application of dsRNA was significant in the reduction of local lesions when 3'dsRNA was used but was not significant in experiments using 5"dsRNA.

Figure 8. Comparison of time of application using 3' and 5' dsRNA



A. Three sets of plants received 3'dsRNA (4 ng/µl) applied to their primary leaves at different time intervals prior to viral challenge. The 1st set received the dsRNA 72 hr prior to viral challenge, the 2nd set 48 hr prior to viral challenge and the 3rd set 24 hr prior to viral challenge. Lesions were counted 72 hr after viral challenge.



B. Three sets of plants received 5'dsRNA (2 ng/µl) to their primary leaves at different time intervals prior to viral challenge. The 1st set received the dsRNA 72 hr prior to viral challenge, the 2nd set 48 hr prior to viral challenge and the 3rd set 24 hr prior to viral challenge. Lesions were counted 72 hr after viral challenge.

Table 3. Time Comparison using dsRNA
The number of viral lesions on plants that received dsRNA at different time intervals prior to viral challenge was compared to each other time interval and to the number of viral lesions on plants that received virus only.

Treatment	Avg. no. viral lesions per half leaf	% reduction in lesions	P value
dsRNA			
72 hr	14.6	62.5	<0.01
48 hr	15.3	31	<0.01
24 hr	27.7	28	<0.01
virus only	39.4		
72 hr compared to 48 hr			ns
72 hr compared to 24 hr			<0.01
48 hr compared to 24 hr			<0.01
5' dsRNA			
72 hr	34.9	80	<0.01
48 hr	50.9	70.7	<0.01
24 hr	76	56	<0.01
virus only	174		
72 hr compared to 48 hr			ns
72 hr compared to 24 hr			ns
48 hr compared to 24 hr			ns

ns = nonsignificant

Effect of multiple applications of dsRNA on inhibition of virus

Experiments were carried out to test the effect of multiple applications of a fixed concentration of 3'dsRNA (18 ng/half leaf) on viral inhibitions. One group of plants received 3 applications of dsRNA 24 hr apart, a second group of plants received 2 applications of dsRNA 24 hr apart and the third group of plants received 1 application of dsRNA. After the final application of dsRNA, the plants were challenged with virus. The application of dsRNA to each group of plants was coordinated so the plants were challenged with virus from the same source. Results of the multiple applications of a fixed concentration of dsRNA reflected reductions in the number of viral lesions at each application level (Figure 9) and several of the plants survived the viral challenge to flower and produce pods (Figure 10).

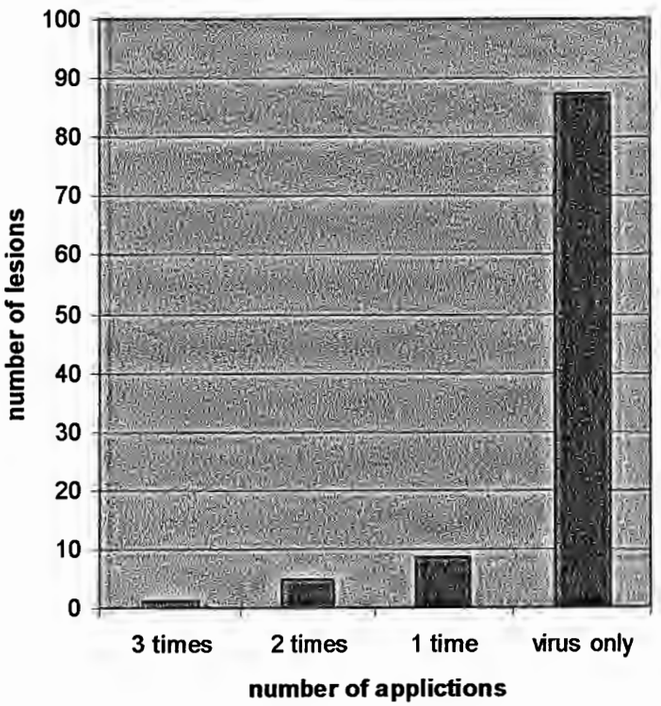


Figure 9. Effect of multiple applications. The same concentration of 3' dsRNA (18 ng/half leaf) was applied 3 times to 1 set of plants, 2 times to a second set of plants and 1 time to a third set of plants in order to achieve a 3X concentration, a 2X concentration and a 1X concentration. There was a 24-hr increment between each application of dsRNA. Virus was applied to all plants 24 hr after the last application of dsRNA. Viral lesions were counted 72 hr after viral inoculation.

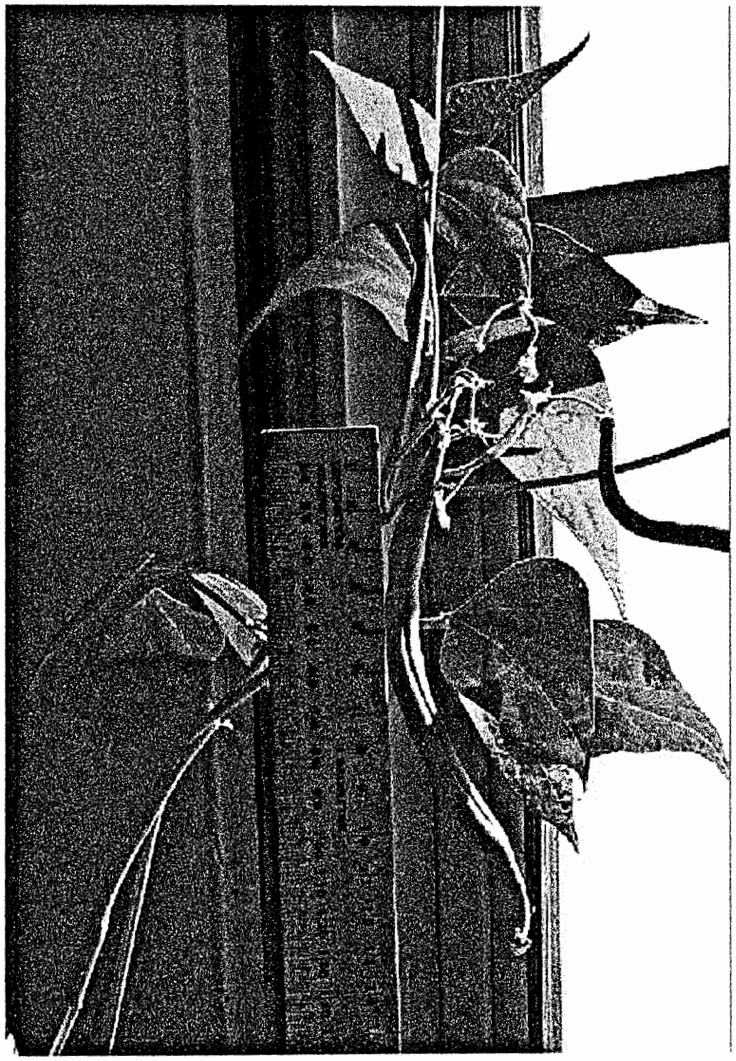


Figure 10. Top Crop bean plant. This plant received three applications of dsRNA prior to viral challenge. The picture was taken thirty-six days after viral challenge. Plant had flowered and produced pods.

Effect of site of homology of dsRNA to genome of BCMNV on inhibition of virus

To determine if the site of homology between the dsRNA and the viral mRNA was a factor, two different segments of dsRNA, each expressing homology to different regions of the BCMNV, were used in this experiment. No significant difference was observed between the 5' dsRNA to the 3'dsRNA on viral inhibition and lesion numbers (Figure 11 and Table 4). While each dsRNA was effective in reducing the number of viral lesions there was no significant difference between the 5' and 3' strands with regards to reduction of viral lesions.

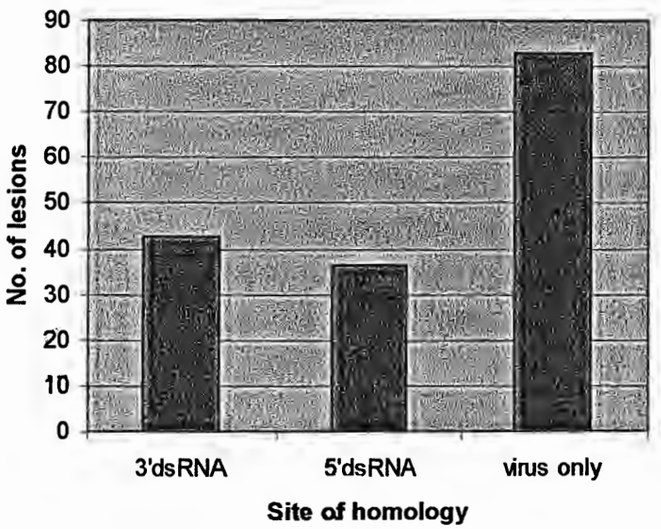


Figure 11. Effect of the site of homology. Double stranded RNA that expressed homology to different regions of the genome of the BCMNV was compared to each other by measuring the number of local lesions. Double stranded RNA (3'dsRNA, 43.5 ng and 5'dsRNA, 20.4 ng) was applied to primary leaves in a half leaf assay and the plant was challenged with virus 24 hr after the application of dsRNA. Lesions were counted 72 hr after viral challenge.

Table 4. Double stranded RNA homologous to BCMNV.
Comparison of the number of viral lesions on plants that received either 5'dsRNA or 3'dsRNA prior to viral challenge.

Treatment	Avg. no. viral lesions	Comparison	P value
3'dsRNA	42.91	3'dsRNA to virus	<0.01
5'dsRNA	36.33	5' dsRNA to virus	<0.01
virus only	83		
		3' dsRNA to 5'dsRNA	ns

ns = nonsignificant

Systemic inhibition of viral replication by dsRNA

The virus initially moves through the plant slowly, cell to cell via plasmodesmata. Once the virus reaches the bundle sheath, it moves into the vascular tissue and then spreads quite rapidly to other parts of the plant. To determine if dsRNA inhibition of viral replication was systemic, 15 µl of either 3'dsRNA (4 ng/µl) or 5'dsRNA (2 ng/µl) were applied to the primary leaf of plants as soon as they reached the required size (average 4096 mm^2). A second set of plants received no dsRNA. The plants were allowed to develop trifoliate leaves (usually 6 to 10 days after application of dsRNA to primary leaves) and the first sets of fully expanded trifoliate leaves were challenged with the virus (Figure 12). The results indicated that viral inhibition was induced by the dsRNA systemically (Table 5).

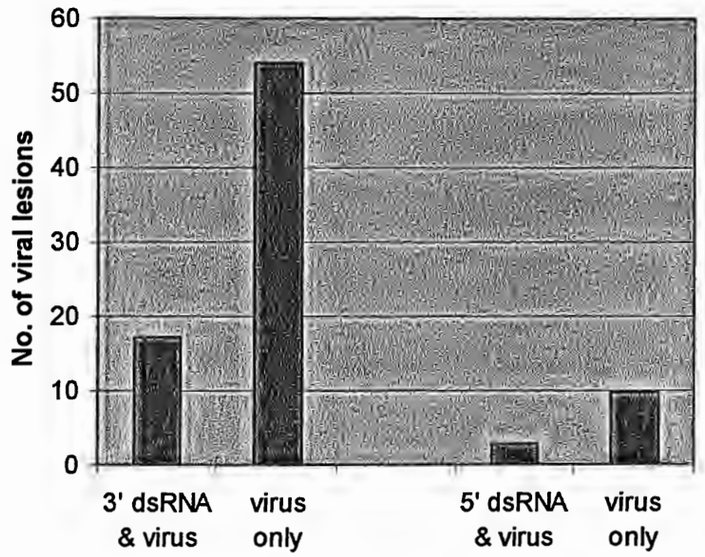


Figure 12. Comparison of viral lesions on trifoliate leaves. Either 3'dsRNA (4 ng/μl) or 5' dsRNA (2 ng/ μl) was applied to primary leaves prior to viral challenge. Virus was applied to trifoliate leaves and viral lesions were counted 72 hr after viral inoculation. The number of local lesions on dsRNA treated plants was compared to the number of local lesions on virus only plants.

Table 5. Comparison of the number of viral lesions on trifoliate leaves
Plants received dsRNA treatment to their primary leaves, trifoliate leaves were inoculated with virus and the number of viral lesions on dsRNA treated plants was compared to plants receiving virus only.

Treatment	Avg. no. viral lesions per half leaf	% reduction in lesions	P value
3'dsRNA & virus	17.21	68.1	<0.01
virus only	54.00		
5'dsRNA & virus	2.7	72.19	<0.01
virus only	9.7		

Viral inhibition induced by dsRNA as a seed borne phenomenon

One of the ways this virus can be transmitted is by seed. The infected plant transmits the virus to its progeny. Since viral inhibition induced by dsRNA was shown to be systemic, the questions arose as to whether the phenomenon of viral inhibition by dsRNA could be passed to the seed and protect the progeny from viral infection. Seeds from plants that had survived viral infection (Figure 13) were germinated and then challenged with virus. Approximately 29% of the seeds failed to germinate. All of the plants that germinated from these seeds died after being challenged with the virus (Figure 14) thus indicating that the phenomenon could not be transmitted to the seeds.

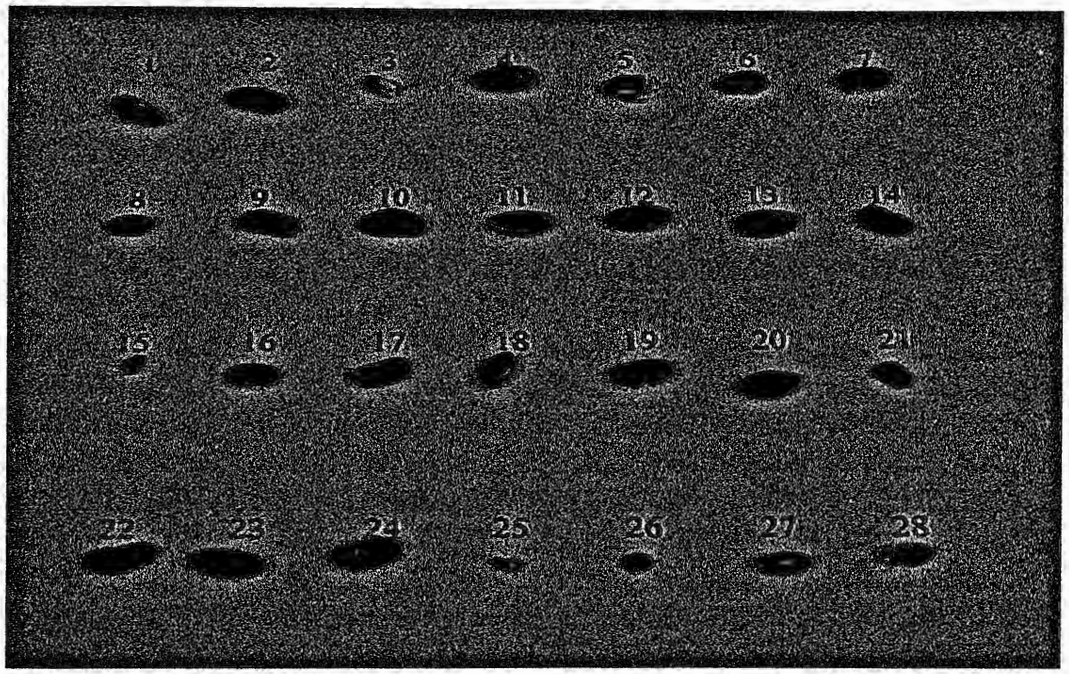


Figure 13. Seeds from plants that exhibited PTGS. Seeds were planted to ascertain if PTGS could be transmitted to seeds.

Figure 14. Plants germinated from seeds from plants that exhibited PTGS. All developed viral lesions after challenged by virus and died within 8 d.p.i.

Isolation of siRNA

Since dsRNA applied to bean plants showed resistance to infection from BCMNV, our hypothesis was the inhibition of virus was due to posttranscriptional gene silencing (PTGS). If PTGS was the explanation for viral inhibition, the siRNA should be present in the plants that exhibited resistance to viral infection. Using standard procedures for isolation of siRNA, no siRNA was detected in primary or trifoliate leaves.

CHAPTER IV

DISCUSSION

This research has shown that dsRNA derived from the BCMNV and mechanically applied to epidermal tissue of primary leaves can interfere with viral infection and reduce the number of local lesions. Other researches (12, 23) have used transgenic plants to confer resistance to plants through a PTGS mechanism. Our research focused on several areas: concentration of dsRNA applied, time of application of dsRNA, multiple applications of dsRNA, applications of dsRNAs that expressed homology to different regions of the BCMNV genome, systemic movement of dsRNA and whether dsRNA could be passed to progeny and confer resistance in them.

Analysis of viral induced gene silencing finds an initiation and maintenance stage (27, 30). The dsRNA intermediate found in viral replication serves to trigger the initiations stage; the mechanical application of dsRNA is analogous to the dsRNA intermediate and served to trigger PTGS.

Our research reflected a 22% average survival rate of plants exposed to the virus. Post-transcriptional gene silencing was expressed about 42% of the time in experiments conducted by Napoli (23) using transgenic plants. The percentage of plants used in this research that received dsRNA and were then challenged with virus that survived to flower and produce seed varied by individual experiment. Survival rates varied from zero

to 70% between experimental groups. There were several factors that may have influenced the survival rate. While Top Crop plants are highly susceptible to lethal infection by BCMNV, some plants may not be as susceptible as others. An example of this may be observed in one of the experiments conducted comparing the concentration of 5'dsRNA on viral infection (Figure 8). The average number of viral lesions on plants that received virus only was 173.75, yet within this group of virus only plants, one plant had as few as 70 lesions/half leaf while another had as many as 279 lesions/half leaf. The virus used to inoculate the plants was extracted from a leaf of virally infected plant just prior to challenging the experimental plants. The virus was in solution and everything possible was done to insure that each mechanical application was identical. One possible explanation for the large discrepancy of viral lesions observed was that individual Top Crop plants vary in the susceptibility to BCMNV.

Discrepancies in the number of viral lesions observed on virus only plants across experiments was also noted. While some of these discrepancies may be due to the individual Top Crop plants involved, an additional explanation may reside in the source of the virus. A reservoir of virally infected Sutter Pink plants was used. One of the characteristics of this virus is to form nuclear inclusion bodies. One of these particles is nuclear inclusion protein b (NIb), the RNA polymerase. This appears to be a mechanism for control of viral amplification (29). Our data (not shown) indicated that newly infected plants yielded more viral particles than older infected plants as older plants have more nuclear inclusion bodies since the virus has already spread from those cells and was not in a state of amplification. This is supported by works of others (20) who observed that in

pulse chase experiments the majority of NIb and CP were found fully processed from polypeptides while other proteins were in an unprocessed polypeptide state; about an hour later the intensity of the bands of the NIb were decreased and the CI protein was fully processed and its bands had increased in intensity. This CI protein is involved in cell to cell movement.

A linear relationship was noted when a viral concentration gradient was compared against the number of local lesions (Figure 5). A similar relationship between different dsRNA concentration and numbers of viral lesions was expected. This relationship was not observed within the concentration parameters used. Our research demonstrated a reduction in viral lesions using various concentrations of dsRNA, but within the parameters used, no difference between concentrations was observed. A possible explanation is the molarity of the smallest concentration of dsRNA was much greater than the molarity of the largest concentration of virus used. It was not possible to assay the concentration of virus used. However a rough estimate of the molarity of the virus used to inoculate the plants was approximately 3.06×10^{-8} pmoles. This is compared to molarity of dsRNA of 2.67 pmoles or more. It would appear that the smallest concentration of dsRNA used was sufficient to trigger PTGS. Anything over that amount was excess. The question arose that if there were a sufficient quality of dsRNA molecules, why did any viral lesions appear on leaves that had received dsRNA? The epidermal tissue of the leaf was abraded with 600-mesh carborendum to allow entry of the dsRNA and the virus into the cell. Attempts were made to cover the surface of the leaf with the dsRNA or virus in a uniform pattern. It is possible that the dsRNA and the

virus were not localized to the same cell during the application process and some virus particle escaped the degradation process.

The vagaries of the analysis of the data left us unable to draw any definitive conclusions. We were able to demonstrate that time of application of dsRNA was a critical factor in reduction of viral lesions and plant survival when using 3'dsRNA. Plants that received the dsRNA earlier rather than later exhibited a significantly greater reduction in local lesions and plant survival rate. However, when using 5'dsRNA the time of application was not a factor. The differences observed were due to chance. It is unlikely that every epidermal cell received dsRNA in the application process. It is also unlikely that the inoculation with the virus put the virus into those same cells. The earlier applications of dsRNA may have allowed some of the dsRNA time to move to adjacent epidermal cells, which may not have received any dsRNA during the application process. Therefore, the greater the time interval between application of dsRNA and viral inoculation allowed for the movement of dsRNA from cell-to-cell resulting in greater viral inhibition as observed by the greater percent reduction in lesions.

We were able to demonstrate that multiple application of dsRNA were more effective than a single application of dsRNA prior to viral challenge. Multiple applications had the effect of delivering to cells an additional concentration(s) of dsRNA as well as offering additional time for movement of the dsRNA between cells. It appeared that the combination of concentration and time had a synergistic effect and significantly reduced the number of local lesions observed on the treated plants.

Our experiments using different strands of dsRNA homologous to different regions of the BCMNV genome produced no differences in results. The molarity of dsRNA used to conduct these experiments was similar; the molarity of the 5'dsRNA and 3'dsRNA molecules was 2.67 pmoles and 3.15 pmoles, respectively. This was an attempt to introduce like number of dsRNA molecules into the leaf. Both strands of dsRNA were able to reduce the number of viral lesions, but there were no differences between the strands. This observation is supported by work done by Tenllado and Diaz-Ruiz (28). Their results using dsRNA homologous to the genome (site for the replicase protein and 30 kDa protein) of the pepper mild mottle virus (PMMoV) to inhibit viral expression yielded data similar to the results observed here.

Our research indicates the dsRNA may move through the plant systemically and confer resistance on leaves that did not receive dsRNA directly. Research by some (12, 17, 24 and 33) supports this observation, but research done by Tenllado and Diaz-Ruiz (28) did not.

The virus can be transmitted from plant to progeny via seed. The virus can either be transmitted by seed if handling contaminates the seed or if the virus infects either the gametes prior to fertilization or invades after fertilization (14). We were unable to demonstrate that progeny from plants that exhibited PTGS were resistant to virus or could inhibit the virus. It appears that the phenomenon could not be transferred via seed. It was doubtful that a mechanism existed to move dsRNA through the plant to the seed.

The application of dsRNA to the primary leaf prior to viral challenge resulted in many plants surviving; they flowered and produced seed indicating that dsRNA did induce PTGS. Closer observation of the plants reflected virus symptoms that seemed to refute this point. One of the plants that had flowered and had just produced a pod exhibited the secondary viral symptoms of leaf curl and a mosaic pattern (data not shown). Four days later viral lesions were observed on the newest trifoliate leaves and the plant subsequently died. To determine if BCMNV was present, a trifoliate leaf from plants that exhibited secondary symptoms, but no viral lesions, was taken. These leaves were triturated and the cell lysate applied to newly grown plants. All of the plants in this group developed local lesions and subsequently died (data not shown). This indicated that virus particles were present in the plants that appeared to be exhibiting PTGS but displayed the mosaic patterns or leaf curl but not local lesions. Many of the plants in this group continued to survive, produced flowers and seeds completing their life cycle. Other plants succumbed to the virus and died. It would appear that while dsRNA applied to members of this group inhibited BCMNV infection, the cell lysate from these plants contained viral particles. Rather than dsRNA inhibiting the virus, it masked the viral infection. This observation is supported by other research (28). Their experiments with dsRNA against the PMMoV reflected the viral multiplication was apparently blocked in leaves inoculated with dsRNA and virus. However, in approximately 20% of their experimental samples, the virus overcame the protection offered by dsRNA and plants displayed the viral disease symptoms one to three weeks after dsRNA/viral inoculation. Even though our experiments indicated dsRNA moved systemically, many plants after

exhibiting inhibition to the virus expressed secondary symptoms. All of these plants were mature plants; they had either flowered and produced seeds or were just about to flower. The plants used to determine if the dsRNA moved systemically were all young plants, less than two weeks old. Research by others (23, 30) sampled plants between 10 and 16 days p.i. for inhibition of virus. A possible explanation for the appearance of PTGS in the plants in this research and the subsequent emergence of viral symptoms was that the application of dsRNA induced PTGS locally in those cells that received dsRNA. In a mechanism not yet completely understood, a mobile silencing signal spread through the plant (30). This signal may have induced the inhibition of the virus in the plant temporarily. However, the dsRNA mechanically applied was eventually degraded by metabolic activity of the plant and the inducement of the silencing signal terminated. Viral particles that penetrated into abraded cells that had not received dsRNA particles escaped the degradation locally and were able to spread and infect upper leaves and synthesize the proteins involved in PTGS silencing. The plant became susceptible to viral infection. The plants that initially exhibited PTGS and then expressed the secondary viral symptoms may have experienced this phenomenon.

It is known that animals and higher plants produce small amounts of dsRNA and dsRNA has been shown to effectively induce interferon. It also appears that the sequence or source of the dsRNA is not important for induction of interferon. The research regarding the function of dsRNA synthesized by plants is in infancy and little is known (17). Plants are able to mount a response to viral infection, particularly against viruses with an RNA genome. This is seen in plants that have an endogenous gene similar in

sequence to the viral genome (27). This differs from interferon inducement by dsRNA. RNA silencing is a general antiviral defense mechanism. Viruses, of course, have evolved a counter defense. They have a suppressor for silencing the silencer in plants (3).

Plants that exhibited PTGS should have contained siRNA. Attempts to assay these plants for siRNA were not successful. Others, applying either ssRNA or dsRNA, have had similar results (28). It may be that the plants initially exhibited PTGS, but the virus was able to overcome the inhibition as outlined above. This may be due to a lack of a nuclear component, which could have affected the accumulation of detectable levels of siRNAs. The maintenance stage of PTGS has been observed in only transgenic plants and may involve the methylation of the transgenic DNA (9).

Applications of dsRNA have been shown to effectively initiate PTGS. The dsRNA needs to express homology to the virus in order to be effective. Mechanical application of dsRNA is impractical in the agricultural industry. In order for PTGS to have practical application in control of viruses in plants, a continuous source of dsRNA homogenous to the viral genome must be available to the entire plant. Given today's technology, this will require transgenic plants. This, of course, gives rise to a polemic social question, which will not be discussed here. Genetically engineered plants should provide the mechanism for viral inhibition, the endogenous gene. The dsRNA-mediated inhibition would be expressed in the entire plant and would also allow for transmission of viral inhibition to progeny.

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