AN ABSTRACT OF THE THESIS OF

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Title: Isolation of the NIa protease gene in wheat infected with wheat streak mosaic virus

Abstract approved: Billie L. Johnson

The nuclear inclusion "a" (NIa) protease gene from three different strains of wheat streak mosaic virus (WSMV) was isolated from WSMV-infected wheat using custom-designed oligonucleotide primers and amplified by polymerase chain reaction. Isolated DNA was cloned into TOP10F' *E. coli* cells after ligation into the pCR®-TOPO plasmid. Transformed TOP10F' cells were visualized by blue/white screening on LB-cb50 medium and ligation was verified by enzyme digestion with EcoR1. Nucleotide sequences of the NIa gene from the different WSMV strains were determined from purified pCR®-TOPO plasmid DNA with the NIa gene inserted. Identity of the cloned gene was determined by sequence analysis with an on-line gene screening databank. The NIa nucleotide sequence was 1268 bases long and began at base # 5424 in the open reading frame of the WSMV genome. The NIa nucleotide sequences from the three different WSMV

strains were 97.3% similar. EcoR1 restriction sites were found within the NIa protease gene from strains H88 and H94PM of WSMV but not from PV57.

ISOLATION OF THE NIa PROTEASE GENE IN WHEAT INFECTED WITH WHEAT STREAK MOSAIC VIRUS

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The true student will seek evidence to establish fact rather than their own perception of truth; truth exists whether it is discovered or not.

Calvin

CHAPTER ONE

INTRODUCTION

General Background

Wheat streak mosaic virus (WSMV) is the most important pathogen of wheat (*Triticum aestivum L.*) in western Kansas and also has been reported in central and eastern Kansas (4). Originally described by McKinney (17) in 1937, it continues to be an economically significant virus in the Great Plains region. Estimated losses from WSMV in Kansas from 1976 to 1987 were as high as 13% of the value of the crop with an average 2.5% of the wheat crop value (14, 21, 30). This becomes even more significant when it is realized that the value of the statewide wheat crop is approximately $1 billion annually (3). It is estimated that WSMV-resistance in wheat would save $350 million nationwide (9).

Amino acid similarity with other potyviruses supports the inclusion of WSMV within the **potyvirus** group of plant pathogens (19, 31). WSMV, as other potyviruses, is a single-stranded RNA (ssRNA) virus encapsulated with a coat protein (31). WSMV, as does other potyviruses, replicates within the cytoplasm of infected plant cells with the help of several viral proteases which act to cleave the translated polyprotein into functional protein fragments. The nuclear inclusion "a" (NIa) protease cleaves the polyprotein at specific sites. These cleavage products are

instrumental in the synthesis of progeny viral particles from the original strand of virus.

At the present time, there are no known inhibitors of the NIa protease in WSMV, resulting in no known lines of wheat immune to WSMV. If an inhibitor to the NIa protease gene were developed and expressed in transgenic mutated wheat, the result would be a line of wheat immune to WSMV. Before such an inhibitor can be developed, the nucleotide sequence of the NIa protease gene must be known. The effectiveness of such an inhibitor would depend on its ability to recognize the NIa protease from different isolates of WSMV (9). Although the complete genome of one strain of WSMV has been sequenced (29), the extent of variation of the NIa protease sequences among various isolates and strains of WSMV is unknown.

Origin of viruses

Different theories have been proposed to explain the origins of viruses. The regressive theory of virus origin proposes that viruses are degenerate forms of intracellular parasites resulting from a microorganism that became critically dependent upon a metabolite that could not pass through a cell membrane. Once inside, such a parasite could progressively lose other biosynthetic abilities without affecting its selection. The end result would be a small

DNA molecule or plasmid that replicates as an obligate intracellular parasite (7).

It is also possible that viruses originated from DNA or RNA cellular components that gained the ability to replicate autonomously and thereby evolve independently. DNA viruses could have arisen from plasmids while RNA viruses could have arisen from self-replicating mRNAs (7).

Enzymatic activity of RNAs suggest an RNA-based evolving system that preceded the current DNA-RNA-protein lifestyle (7). The reactivity of RNA, which favors its use in catalysis, makes it a poor molecule for storage of genetic information. This theory suggests that self-replicating RNAs co-evolved along side DNA-based cells and parasitized these hosts, eventually becoming the RNA viruses (7).

Factors affecting RNA virus evolution

RNA viruses can infect prokaryotes as well as eukaryotes. RNA viruses seem to be a special class of mRNA molecules (introns are lacking among the RNA viruses) that have evolved mechanisms for replication. Eukaryotic cells employ enzymes known as RNases to control the longevity of mRNAs. These RNases have made it difficult to isolate intact RNA viral genomes from cells (7).

The rate of divergence of RNA viruses is very rapid.

The rate of divergence of RNA viral genomes at the nucleotide level is 0.03% to 2.0% per nucleotide per year, a rate about one million times the rate for eukaryotic DNA genomes (7).

Studies of RNA viruses have also been hampered by the extreme virulence of many isolates, inherent difficulties in direct sequencing of RNA, and the failure of most RNA viruses to undergo recombination. The advent of cDNA cloning and of the sequencing of RNA viral genomes as plasmid copies has enormously increased the potential for learning about RNA viruses.

Virus characteristics

Virus particles have only recently been characterized with regards to their physical characteristics. As a group, they are smaller than the smallest bacteria, which prevents light microscopy from being used as a diagnostic tool. Virus particles have linear dimensions measured in nanometers (10^{-9} m) and are visible only by electron microscopy (23). Molecular mass of virus particles has a range between 100,000 to 100 million atomic mass units, although the mass of individual component sections of the virus particle is more commonly reported as kilodaltons (kDa) (15).

Virus particles have two basic structural shapes;

helical, which has a rod-like appearance, and isometric, which has a spherical shape (15). The minimal structure of a typical virus consists of one molecule of nucleic acid surrounded by a protein shell or coat. More complex viruses may contain several molecules of nucleic acid as well as several different proteins, internal bodies of definite shape, and complex envelopes with spikes that usually contain glycoproteins and lipids (15).

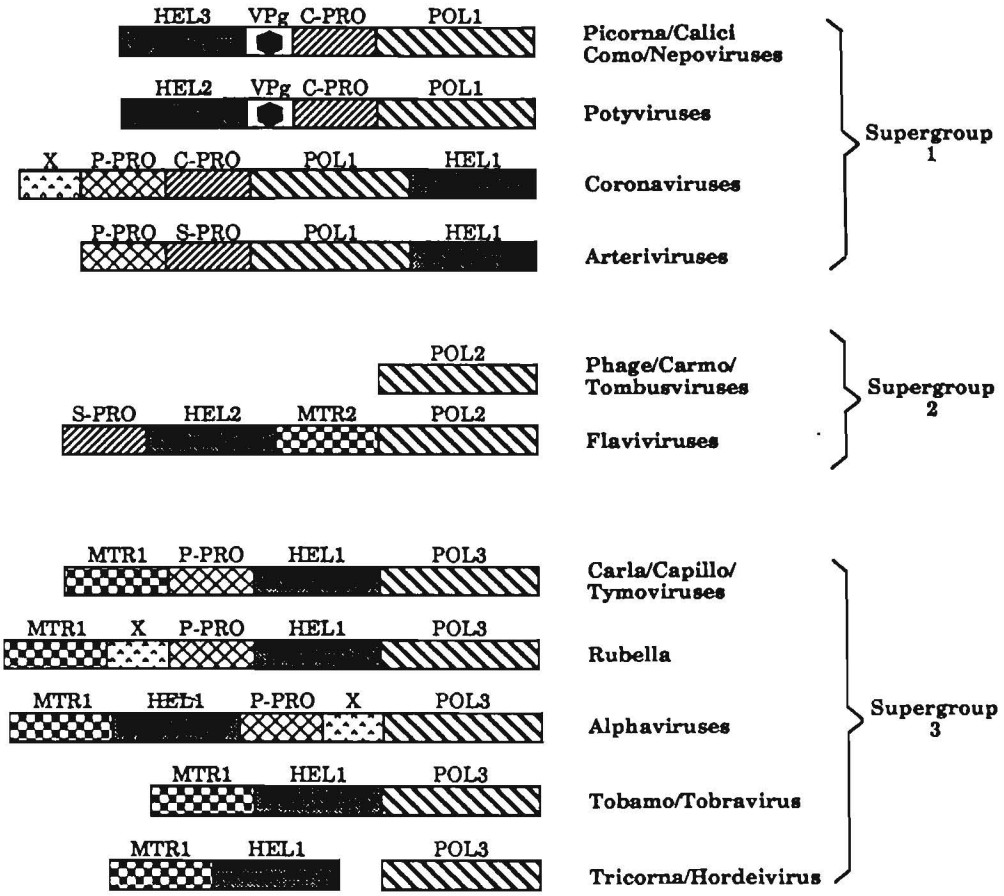
The complete virus particle is called a virion. The term "virus" differs from "virion" in that "virion" refers only to the inert or nearly inert particle while "virus" consists of all virus-specified entities involved in all aspects of the infection cycle. Viruses are generally considered to be non-living entities, although they do have some characteristics of living cellular organisms: a life cycle with defined stages, a high state of organization, and genetic material (15). Viruses do not possess the cellular organelles necessary for metabolism such as protein synthesis. They cannot reproduce outside a host cell. Viral genes are able to redirect a cell's metabolism to produce virus-specified products. The information-bearing molecule in a virion is either RNA or DNA but not both. Upon entering the nucleus of a suitable cell, the viral genomic nucleic acid redirects the genetic and metabolic apparatus of the cell to produce new virons (15).

Potyviridae characteristics

Over 30% of all plant viruses belong to the Potyviridae family, making it the largest of all plant virus families (20). Differences in virus transmission have been used to designate three genera: Potyvirus (aphids), Bymovirus (fungi), and Rymovirus (mites) (7). RNA viruses can also be divided into three supergroups or families based on the arrangement of a small number of genes for replicative functions (Fig. 1). Potato virus Y is the type species of the potyviruses but tobacco etch virus (TEV) has been more extensively characterized. Potyviruses are considered the most destructive of the plant viruses. Most of the world's important vegetable and field-crop growing areas sustain serious losses due to these viruses (20).

Virions of potyviruses are flexuous rods 11 to 15 nm in diameter and 680 to 900 nm in length. The potyviral genome is ssRNA of about 9 - 10 kilobases (kb) and has a 5' terminal genome-linked protein (VPg) and a 3' polyadenylated tail (20). The RNA contains a single open reading frame (ORF) encoding a polyprotein of about 340 kDa that is processed by three virus-encoded proteinases (P1, HCpro, and NIa). The serine-like NIa proteinase, cleaves itself from the polyprotein and performs several other processing reactions (7).

Figure 1. Diagram of Supergroups of RNA viruses based on arrangement of genes for replicative functions such as; helicase (dark gray), genome-linked protein (hexagon), chymotrypsin-like proteinase (fine diagonals), polymerase (bold diagonals), papain-like proteinase (crosshatch), methyltransferase (checkerboard), and a region of unknown function (stippled). Reprinted from Fields, et al. (7).


HEL3 VPg C-PRO POL1
Picorna/Calici Como/Nepoviruses
HEL2 VPg C-PRO POL1
Potyviruses
X P-PRO C-PRO POL1 HEL1
Coronaviruses
P-PRO S-PRO POL1 HEL1
Arteriviruses
Supergroup 1
POL2
Phage/Carmo/ Tombusviruses
S-PRO HEL2 MTR2 POL2
Flaviviruses
Supergroup 2
MTR1 P-PRO HEL1 POL3
Carla/Capillo/ Tymoviruses
MTR1 X P-PRO HEL1 POL3
Rubella
MTR1 HEL1 P-PRO X POL3
Alphaviruses
MTR1 HEL1 POL3
Tobamo/Tobravirus
MTR1 HEL1 POL3
Tricorna/Hordeivirus
Supergroup 3

WSMV characteristics

WSMV causes a systemic infection that can be recognized by a yellowish-green mosaic pattern on the leaf surface (Fig. 2). The mosaic pattern consists of yellow-green dashes and streaks usually limited by the larger leaf veins (11). Other symptoms include stunting and necrosis resulting in subsequent reduction of yield (27). WSMV is transmitted by the wheat curl mite (*Aceria tosichella* Keifer) (1, 13), whereas most potyviruses are aphid-transmitted (19).

The WSMV virion is a flexuous rod of approximately 700 x 13 nm as determined by electron microscopy in 1953 (8). WSMV is encapsulated by a capsid protein with a relative molecular mass ranging from 45 to 48 kDa (23, 26). Although early reports of viral instability made purification difficult, Brakke published one of the first routinely successful purification protocols in 1958 (2).

Tolerance to WSMV infection has been shown in the wheat cultivar, Triumph 64 (24). High levels of resistance to WSMV has been recently incorporated into wheat (25). This source of resistance is not associated with adverse agronomic characteristics and may provide a highly effective source of WSMV resistance. However, this does not lessen the need for additional sources of resistance because this new level may succumb to WSMV mutations. Biological

Figure 2. Wheat mechanically infected with wheat streak mosaic virus.

variation in WSMV isolates has recently been demonstrated (26). In addition to wheat, WSMV has been isolated from corn, sorghum, and pearl millet (22).

In the past decade, a concerned effort has been made to determine the genome structure of WSMV. Niblett, et al., (19) have sequenced the capsid protein gene. However, difficulty in attaining pure virus and maintaining intact RNA *in vitro* has made sequencing of the viral genome problematic. Niblett, et al., (19) also found the presence of an unidentified N-terminal blocking group that prevented sequencing of the full-length capsid protein. The complete genome of WSMV has only recently been successfully sequenced (29).

Once the coat protein is removed, the RNA strand is extremely susceptible to degradation by RNases. When extracted WSMV-RNA is subjected to electrophoresis on agarose gels, RNase activity will cause a collection of small, variable-sized viral particles instead of one intact strand of viral RNA.

Viral infection cycle

Viruses differ greatly in their mode of infection of the host cell. Most plant viruses are dependent upon mechanical damage to the cell wall before they can enter. Such damage results from physical forces (wind, abrasion),

but more frequently the virus is introduced into the cell by a particular animal vector (insects, mites, nematodes), or by a soil-borne fungus through the roots. Infection, once started, spreads in plants largely through the abundant plasmodesmata connecting the hair cells and the lower epidermal cells (15).

There are several distinct stages of infection (Fig. 3). The earliest stage is generally called **adsorption and entry** during which virions enter the host and attach to the cell, allowing the viral nucleic acid to enter the cell. Initial interaction of virion and cell occurs through random collision, therefore a high concentration of virus and cell facilitates rapid initiation of infection in cells.

During the next stage of infection, the **latent period**, the main processes of virus replication occur: production of viral nucleic acids and proteins. Within the latent period is the **eclipse period** which lasts only until new virions are formed inside the cell. During the eclipse period, infectivity is not detected. The period of **assembly** of virions from their structural components and nucleic acids occurs late during infection, and the final steps of **maturation** of virions in infectious form may continue even during their release from the original virion from the infected cell. The release of new virions is commonly called the **burst**, indicating the sudden disruption of the

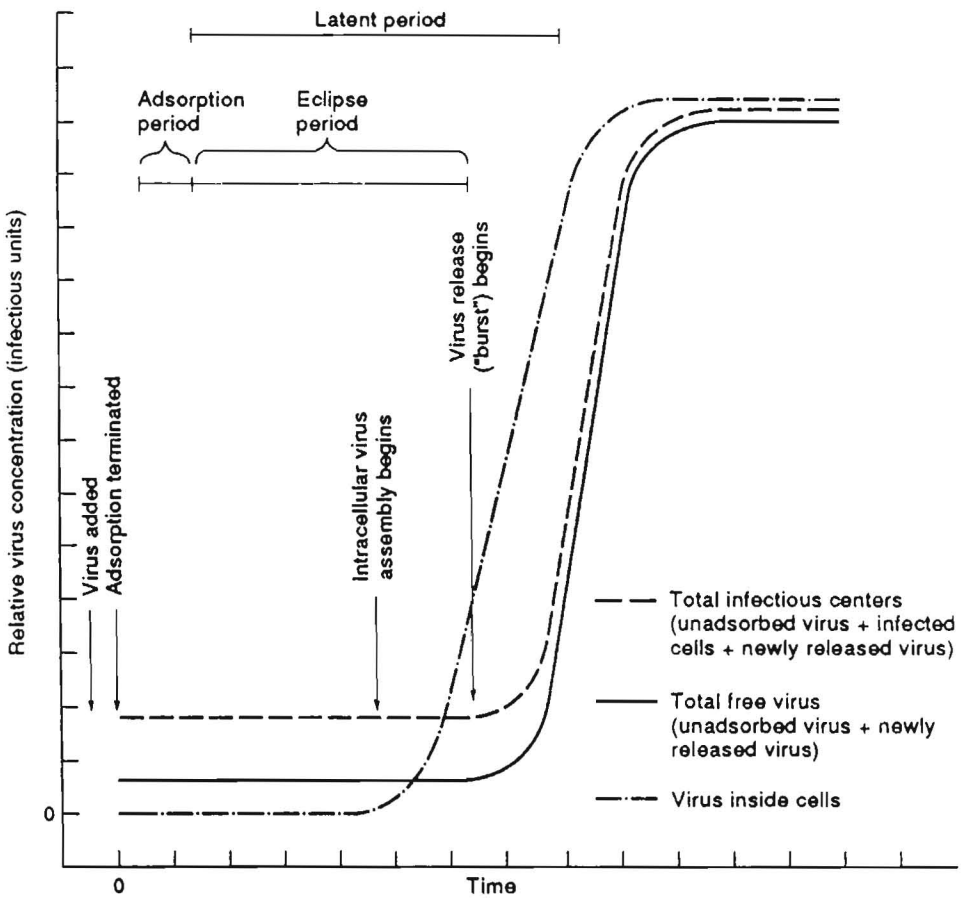
infected cell. This event may take a few minutes for bacterial viruses or several hours or days for eukaryotic viruses. The main reason for this difference in time scales may be the sheer enormity of animal and plant cells in comparison with bacterial cells (perhaps a 1000-fold difference in volume) (15).

During the replication cycle, viruses generally produce identical copies of themselves but errors resulting from the action of their DNA or RNA polymerases and other causes, yield viruses that contain genetic mutations. In general, RNA viruses are more error-prone in their replication than DNA viruses. An RNA mutational frequency as high as 10^{-4} to 10^{-5} base pair substitutions per single base site have been observed while DNA viruses generally mutate at a 10^{-7} to 10^{-11} frequency (15).

Role of NIa protease gene in proteolytic cleavage

Potyviruses replicate in the cytoplasm, but two polypeptides released by proteolytic cleavage of their polyprotein precursors (the 49 and 58 kDa proteins, also known as NIa and NIb, respectively) accumulate as nuclear inclusion bodies. Figure 4 shows a schematic of the genome organization of both tobacco etch virus and turnip mosaic virus. The NIa protease gene is responsible for several cleavages of the polyprotein. The 49 kDa (NIa)

Figure 3. Generic viral reproductive cycle. Reprinted from Levy, et al. (15)


Latent period
Adsorption period
Eclipse period
Virus release ("burst") begins
Intracellular virus assembly begins
Virus added
Adsorption terminated
Relative virus concentration (infectious units)
0
0
Time
Total infectious centers (unadsorbed virus + infected cells + newly released virus)
Total free virus (unadsorbed virus + newly released virus)
Virus inside cells

protein is a serine-like protease, whereas the 58 kDa (NIb) protein exhibits certain sequence similarities to other viral RNA polymerases (15). Replication of WSMV in a plant cell is thought to be similar to that of other potyviruses in that highly specific proteases cleave viral polyproteins into smaller proteins of which several function as different types of polymerase proteins (6). These protein fragments subsequently play a role in additional strands of viral RNA being synthesized from the original "plus" strand of WSMV. Wheat mechanically inoculated with WSMV will begin to exhibit visible mosaic symptoms between five to ten days after inoculation (16).

Potyviruses induce the formation of characteristic cylindrical inclusions in the cytoplasm of infected cells. These inclusions are complex structures containing a central tubule from which radiate curved "arms" to give a "pinwheel" appearance. These inclusion bodies are formed from a 70 kDa virus-encoded polypeptide that contains the highly conserved nucleotide triphosphate-binding motif present in most viral RNA-dependent RNA polymerases. The central tubule is located directly over the plasmodesmata; cytoplasmic connections through the plant cell wall. The cylindrical inclusion may continue from cell to cell. Virus particles are closely associated with these inclusion bodies,

particularly early in infection (15).

Sequence analysis

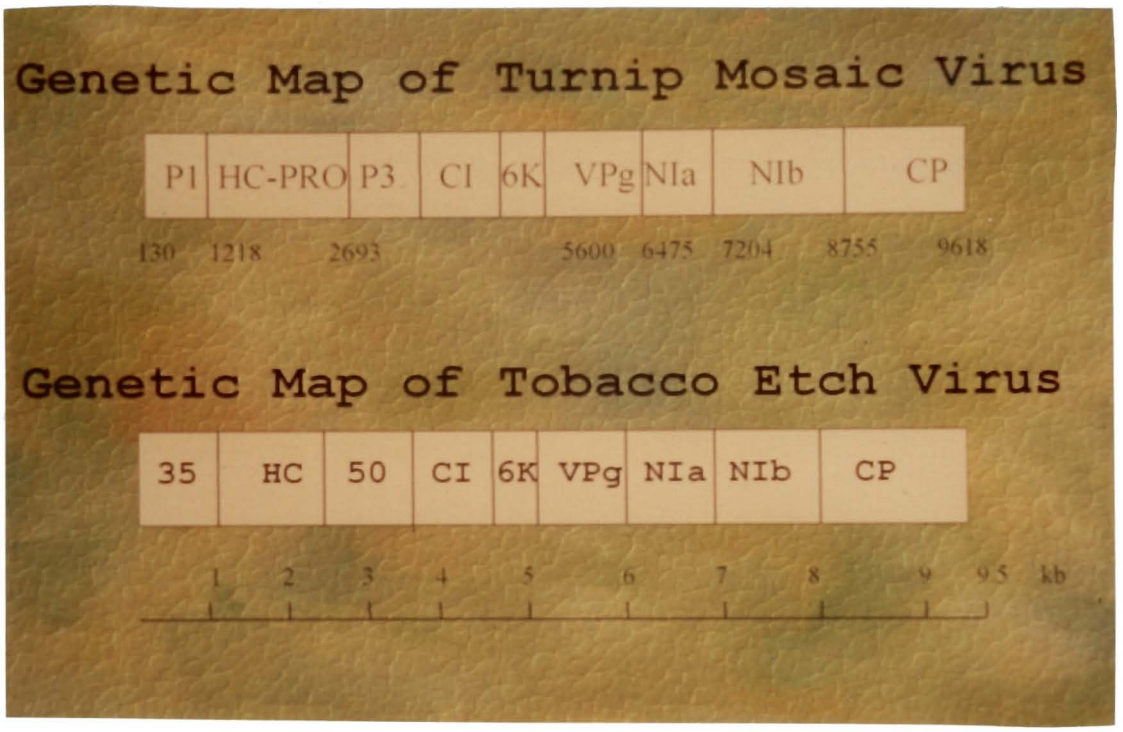
A prerequisite for detailed analyses of viral functions is the availability of sequence information. Single-stranded nucleic acids that have sequences encoding proteins are considered to be of positive polarity (also known as the "plus" strand). The string of nucleotides beginning at an AUG start codon and ending just before the stop codon is an open reading frame (ORF). An ORF is a potential polypeptide-encoding region of the molecule (15).

Long translational ORFs uninterrupted by stop codons predicted from the DNA sequence suggests the presence of protein-coding sequences (7). Methionine codons used to initiate translation are found at the beginnings of ORFs (7). Sites of mRNA cleavage and polyadenylation are almost always signaled by the sequence, AAUAAA (24). In eukaryotes the starts of transcription by RNA polymerase II are usually about 25 basepairs (bp) downstream of "TATA" boxes (7).

Expression of viral genes in heterologous systems

It is often difficult to test the functions of certain viral proteins in the absence of other viral or cellular proteins because their low abundance in virus-infected cells

Figure 4. Organization of the turnip mosaic virus and tobacco etch virus genome. Reprinted from Fields, et al. (7).


Genetic Map of Turnip Mosaic Virus
P1
HC-PRO
P3
CI
6K
VPg
NIa
NIb
CP
130
1218
2693
5600
6475
7204
8755
9618
Genetic Map of Tobacco Etch Virus
35
HC
50
CI
6K
VPg
NIa
NIb
CP
1
2
3
4
5
6
7
8
9
9.5
kb

prevents their purification. Recombinant DNA technologies have offered solutions to these problems by allowing the expression of cloned viral genes in heterologous systems.

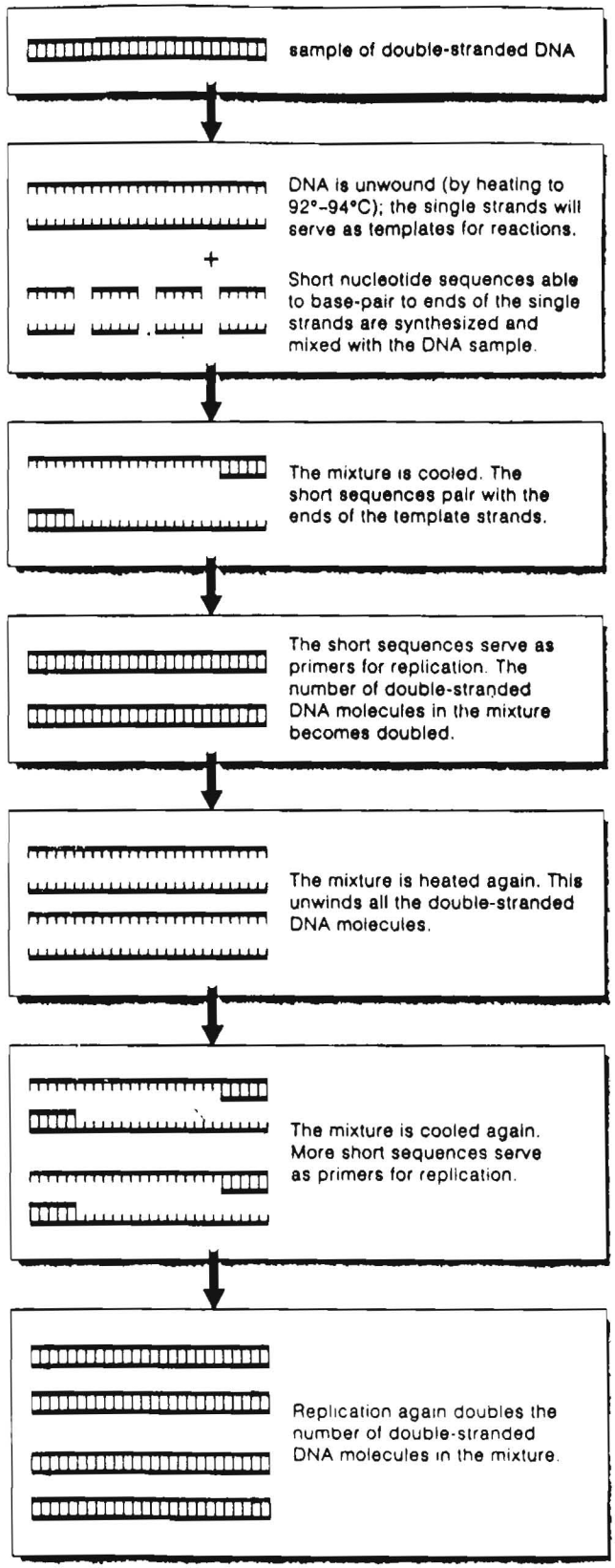
Escherichia coli (*E. coli*) is a useful heterologous expression system. It is possible through the use of ligation enzymes to clone viral genes into plasmids which serve as expression vectors. Transformed plasmids can then be expressed in competent *E. coli* cells as fusion proteins, in which viral coding sequences are fused to a prokaryotic control sequence such as the *lacZ* gene, which encodes β-galactosidase or specific antibiotic resistant genes. Growth of transformed *E. coli* on selective media is evidence the transformed plasmid has been successfully cloned by the *E. coli* cells (15).

Protease activity

Without active proteases, WSMV cannot replicate. Most viral proteases are genetically related and are structurally similar to the trypsin-like family of proteases, which are ubiquitous in nature (9). Potyviruses, however, have a different amino acid (cysteine) in their active sites than the trypsin-like proteases (serine) (9). This difference prevents known inhibitors of trypsin; chymotrypsin, or elastase from binding to, and inhibiting potyvirus protease

(9). For an inhibitor to be active, it must have both the proper reactive site and a surface complementary to the surface of the protease (9). Tobacco etch potyvirus (TEV) nuclear inclusion body "a" gene (NIa) (also known as 49 kDa protease), a highly conserved gene among the potyvirus proteases, is responsible for cleavage at five sites in the TEV polyprotein (6). When WSMV infects a cell, the NIa protease initiates a cleaving action at specific sites on the polyprotein, thus allowing the virus to continue replication. As NIa continues to cleave the translated polyprotein, the number of virions increases exponentially. Before an inhibitor to NIa can be engineered, the protease must be identified, isolated, and sequenced. The isolation of NIa in turnip mosaic potyvirus has been successful (18). Oligonucleotide probes having complementary sequences both upstream and downstream from NIa could be used to provide evidence that the NIa protease gene has been located and could subsequently be amplified by reverse transcription followed by a polymerase chain reaction (RT-PCR) (10). Figure 5 shows flow chart of a basic polymerase chain reaction.

Figure 5. A flow chart of a polymerase chain reaction following reverse transcription of single-stranded RNA. Reprinted from Starr and Taggart (28).


sample of double-stranded DNA
DNA is unwound (by heating to 92°–94°C); the single strands will serve as templates for reactions.
+
Short nucleotide sequences able to base-pair to ends of the single strands are synthesized and mixed with the DNA sample.
The mixture is cooled. The short sequences pair with the ends of the template strands.
The short sequences serve as primers for replication. The number of double-stranded DNA molecules in the mixture becomes doubled.
The mixture is heated again. This unwinds all the double-stranded DNA molecules.
The mixture is cooled again. More short sequences serve as primers for replication.
Replication again doubles the number of double-stranded DNA molecules in the mixture.

Research Objective

The purpose of this study is to determine if there is biological variation of the NIa protease gene among three isolates of WSMV. It is designed to identify, isolate, and sequence the NIa gene of WSMV. Biological variation will be determined by comparing the NIa protease nucleotide sequence between three different WSMV isolates. The NIa protease gene will be subsequently cloned by transforming plasmids of a maintenance vector with isolated cDNA from the NIa protease gene. It is hoped that future work will include the engineering of an inhibitor to the NIa protease, effectively blocking its cleaving ability. Ideally, this inhibitor can be transformed into the genome of millable wheat. If this proves successful, genetically engineered wheat resistant to WSMV will be commercially available for the first time.

CHAPTER 2

MATERIALS AND METHODS

Virus Maintenance

Three isolates of WSMV were obtained from Dallas Seifers at the KSU Agricultural Research Center-Hays and maintained in Arkan wheat in a greenhouse facility at Kansas State University. Arkan wheat is susceptible to WSMV with only mild stunting of wheat plants, making Arkan a good variety in which to maintain the virus. Temperatures in the greenhouse ranged between 25 to 30 C during the months of June and July. No artificial lighting was used. Wheat seedlings were finger-inoculated on the second or third leaf with a 1:5 (w/v) extract of infected wheat tissue macerated in a 0.02 M K_2HPO_4 buffer (pH 6.8) with carborundum (600 mesh) added to a 1% concentration to facilitate virus infection. Infected wheat was harvested and used immediately when wheat was between 10 to 14 days post-inoculation or stored in plastic zip-lock bags at - 20 C to be used at a later date. Karr and Seifers showed WSMV remains as infective as freshly prepared extract when stored *in vivo* at -20 C for 14 days (12).

Healthy wheat was maintained in the same greenhouse to serve as a check for cross-contamination between virus isolates and also as a healthy comparison. Planting

schedules were maintained such that there was same-aged healthy wheat harvested each time infected wheat was harvested.

The three strains of WSMV used in this study were PV57, H88 and H94PM. All isolates produced similar symptoms although PV57 caused the most pronounced mosaic symptoms and the most severe stunting. WSMV-PV57 was isolated in Saline County, KS in 1932 and is considered to be the virus type speciman for WSMV. WSMV-H88 was isolated in Hays, KS in 1988. WSMV-H94PM was isolated in Hays, KS in 1994. It is of considerable interest to note that H94PM was isolated out of pearl millet. Serological similarities were verified by indirect ELISA by Seifers (22).

ISOLATION OF TOTAL RNA

Diseased and healthy wheat (14 to 28 days post inoculation) were used for RNA extraction. The two youngest leaves, approximately 0.1 g of leaf tissue, from three plants inoculated with one of the three strains showing obvious mosaic symptoms were removed from the plant. These were placed in a 1.5 mL microfuge tube and immediately put on ice. The tissue was freeze-dried with liquid nitrogen, crushed in the microfuge tube with a steel pestle and treated with 1.0 ml of TRIzol reagent (Gibco BRL, Grand Island, NY). TRIzol is a phenol/guanidine isothiocyante

solution which maintains the integrity of RNA while disrupting the cell. The tissue/TRIzol mixture was well mixed and incubated at room temperature (RT) for 5 minutes to allow for complete dissociation of nucleoprotein complexes. Freezer-cold (-20 C) chloroform (0.2 ml) was added to each tube, mixed by shaking the tube for 15 seconds and incubated at RT for 3 minutes. Chloroform causes the mixture to separate into a lower, phenol-chloroform phase and a colorless, upper aqueous phase. RNA remains exclusively in the aqueous phase. The solution was centrifuged in an Eppendorf 5415 C microcentrifuge at 11,750 x *g* for 15 minutes at 5 C. The aqueous phase (approximately 0.6 ml) was removed with a pipette and placed in a new microfuge tube. Freezer-cold isopropanol (0.5 ml) was added to each tube, mixed by shaking the tubes for 15 seconds and incubated at RT for 10 minutes. The RNA was pelleted by centrifugation at 11,750 x *g* for 10 minutes at 5 C. The supernatant was immediately decanted and RNA pellets were washed in 1.0 ml of freezer-cold 75% ethanol prepared in water treated with diethylpyrocarbonate (DEPC). DEPC is an inhibitor of RNases. The RNA was repelleted by centrifugation at 6,610 x *g*) for 5 minutes at 5 C. After the supernatant was decanted, tubes were inverted and pellets were allowed to air-dry at RT for 5 minutes. The dried RNA pellets were dissolved in 0.1 ml of DEPC-water by

trituration with a pipette tip, and incubated in a 55 C water bath for 10 minutes. RNA concentration (µg / µl) of each sample was determined by making a 1:100 dilution (10 µl RNA sample added to 0.99 ml of DEPC-water) and recording the UV absorbance readings at wavelengths of 260 and 280 nm. The integrity of the original RNA preparation was determined immediately by electrophoresis. Ideally, the viral RNA will be visible as a separate and unique band, however this is unlikely because of the abundance of ribosomal RNA present. The RNA sample was stored at -20 C but degradation after one week made long-term storage of RNA impractical.

RNA electrophoresis conditions

Gels used for RNA analysis were prepared by dissolving 0.500 g of high melting (HM) agarose in 50 ml of 1x MOPS buffer. This mixture was heated in a microwave for approximately 90 seconds, or until the agarose completely dissolved, and allowed to cool before pouring into a 10 x 6.5 x 0.5 cm gel cast. Just prior to pouring, 2.5 µl of ethidium bromide (EtBr) (10 mg / ml) was added to the agarose solution. EtBr is an intercalating compound which causes nucleic acids to fluoresce under UV light. The running buffer consisted of 270 ml of double-distilled water ($_{dd}H_2O$), 30 ml of 10x MOPS buffer and 12 µl of EtBr.

Electrophoresis of RNA was carried out at 90 volts and 50 to 60 milliamps for approximately 90 minutes. All electrophoresis runs were performed at RT. Prior to loading the RNA sample onto a gel, 20 µg of each RNA sample was placed in a new 0.5 ml microfuge tube and mixed with 1.6 µl of 10x MOPS, 3.2 µl of formaldehyde, and 9.0 µl of formamide. This solution was then incubated at 60 C for 10 minutes. After incubation, 4 µl of 10x bromophenol blue loading buffer was added to each tube. Individual wells in the gel were loaded with 20 µl of each RNA preparation. A 9.47 - 0.24 kilobase (kb) RNA ladder (Gibco, BRL) was used as a marker. Preparation of the RNA ladder was identical to that of the RNA samples with the exception that 5 µl of RNA ladder was used in place of 20 µg of RNA.

DNA electrophoresis conditions

DNA samples were analyzed on 1% HM agarose gels. Gels used for DNA analysis were prepared by adding 0.5 µg of HM agarose to 50 mL of $_{dd}H_2O$ and adding 1.0 ml of 50x TAE buffer. This mixture was heated in a microwave for approximately 90 seconds, or until the agarose was completely dissolved, and allowed to cool before pouring into a 10 x 6.5 x 0.5 cm gel cast. Prior to pouring, 2.5 µl

of EtBr was added to the cooled agarose solution. The running buffer was prepared by diluting 6 ml 50x TAE in 300 mL $_{dd}H_2O$ and adding 12 µl EtBr. Samples of DNA preparations were mixed with an equal volume of $_{dd}H_2O$ (usually 10 µl DNA was mixed with 10 µl of $_{dd}H_2O$ although this ratio could change depending upon the concentration of DNA). Prior to loading, 4 µl of a 5x xylene loading buffer was mixed with the DNA sample. Individual wells were loaded with 20 µL of the prepared DNA sample. A 1 kb DNA ladder (Gibco, BRL) was used as a marker. The DNA ladder was treated the same as the DNA samples except 2 µl of 1 kb DNA ladder was mixed with 18 µl of $_{dd}H_2O$. DNA samples were subjected to electrophoresis at 90 volts and 50 to 60 milliamps for 90 minutes.

NIa protease gene isolation by PCR

Isolation of the NIa protease gene template in the total RNA preparation was accomplished by using the Titan™ One Tube RT-PCR System (Boehringer Mannheim, Indianapolis, IN). The simplicity of this system is that it allowed cDNA of the RNA template to be synthesized and subsequently amplified by PCR without addition of reagents between cDNA synthesis and PCR. This system involves a solution of upstream and downstream primers for the RNA template, dNTP

cocktail, AMV reverse transcriptase, and *Taq* DNA polymerase all in an optimized RT-PCR buffer.

Custom primers were synthesized by the Biochemistry Department at Kansas State University. Hairpin and palindrome analyses were performed prior to ordering by using a primer analysis program on the Internet: **[http://www.cybergene.se/primer.html].** Lyopholized primers were resuspended in TE buffer to bring stock solutions to 100 pmol / µl and stored at -20 C. Working solutions of primers (10 pmol / µl) were prepared by diluting 3 µl of the stock solution in 27 µl of TE buffer and also stored at -20 C.

The downstream primer was custom designed to be a 30 base oligonucleotide sequence from the 5' side of the adjacent NIb gene:

5'- AGAATCCTTATTGCCAACTAACCAAGTCGC - 3'.

The upstream primer was custom designed to be a 26 base oligonucleotide sequence from the 3' side of the NIa gene sequence from a different WSMV isolate, H94S:

5'- CACGCAACAGGAGTGCAAAACGACAA - 3'.

This unpublished sequence was obtained from

Mr. Dingbing Yang, a graduate student who was also working with WSMV. The length between the upstream and downstream primers was approximately 1.3 kb.

The Titan™ One Tube RT-PCR system was divided into two master mixes. Master mix 1 consisted of dNTPs (1 µl each or 0.2 mM each), primers (2 µL each or 0.4 µM each), template RNA (1 µg -1 pg total RNA), DTT-solution (2.5 µl or 5mM), RNase Inhibitor (0.4 µl or 5 - 10 U), and was brought to a final volume of 25 µl with sterile $_{dd}H_2O$. Master mix 2 consists of RT-PCR buffer (10 µl or 1.5 mM $MgCl_2$), enzyme mix, 1 µl, and was brought to a final volume of 25 µl with sterile $_{dd}H_2O$. A cocktail of master mix 2 was prepared by increasing the volumes of RT-PCR buffer, enzyme mix, and $_{dd}H_2O$ by the number of samples that were to undergo RT-PCR.

Master mix 1 (25 µl) and master mix 2 (25 µl) were combined in a 0.2 ml thin-walled PCR tube placed on ice. The sample was vortexed briefly (approximately 10 seconds) and centrifuged briefly (16,000 x *g* for appoximately 10 seconds) to concentrate the sample at the bottom of the tube. Each sample was overlayed with 30 µl of sterile light mineral oil to prevent evaporation during the RT-PCR reaction. The sample was incubated at 50 C for 30 minutes for cDNA synthesis. After 30 minutes, the sample was subjected to PCR in a thermocycler. The RT-PCR required 3.5

hours to complete. Figure 6 shows the cycle protocol for the RT-PCR. A sample of the amplified cDNA (PCR product) was then analyzed on a 1% high-melting agarose gel that contained EtBr.

Sequence determination of NIa protease gene

cDNA of the amplified NIa gene was isolated by comparing band migration to the DNA ladder used in the agarose gel. The 1.3 kb cDNA band was excised from the agarose gel with a razor blade and transferred to a microcentrifuge tube. The gene was then subjected to a gene cleaning procedure. The QIAEX II agarose gel extraction kit (QIAGEN Ltd., Santa Clarita, CA) was used. Each gel slice was weighed and 3 volumes of QX 1 buffer (supplied in kit) was added (300 µl for every 100 mg of gel). Glassmilk was added to each sample (20 µl of QIAEX II per sample) for DNA adsorption to the silica gel and the tubes were incubated in a 55 C waterbath for 10 minutes. During incubation, the tube contents were mixed by flicking every 2 minutes. After incubation, 2 µl of 3 M $NaCH_3COO$ (pH 5.0) was added to each tube and the Glassmilk/DNA pellet was precipitated by centrifugation at 16,000 x *g* for 30 seconds in an Eppendorf 5415 C microcentrifuge at 5 C. Following centrifugation, the supernatant was decanted and the pellet was resuspended in 0.5 ml of fresh QX 1 buffer. After repelleting,

Figure 6. Conditions for the RT-PCR.

1x	Reverse transcription at 50 C for 30 minutes.
1x	Denature RNA template at 94 C for 2 minutes.
35x	Denaturation of template at 94 C for 30 seconds. Annealing of primers at 50 C for 30 seconds. Elongation of cDNA at 68 C for 45 seconds to 4 minutes. (increase elongation 5 seconds for each cycle after cycle no.10)
1x	Prolonged elongation for 7 minutes.

(30 seconds at 16,000 x *g*), the supernatant was decanted and the pellet was washed twice in PE buffer (supplied in kit). After the final wash, the tubes containing the pellets were inverted and the pellets allowed to air-dry for 20 minutes at RT. The pellets were dissolved in 20 µl of $_{dd}H_2O$ (pH 7.0 to 8.5) and centrifuged (30 seconds at 16,000 x *g*). The supernatant contained the eluted cDNA and was transferred to a new tube. This final procedure was repeated to increase the yield of cDNA and also increased the DNA sample volume to 40 µl. The final concentration of DNA was determined by running 4 µl of each DNA sample on a 1% agarose gel along with 4 µl of a Low DNA Mass Ladder. Concentration of DNA was determined by comparing band intensity of each DNA sample to that of the bands in the Low DNA Mass Ladder of known concentrations. A sample of the cleaned DNA for each isolate along with a sample of both PCR primers was sent to the DNA Sequencing Facility at Iowa State University, Ames, IA.

Cloning of NIa gene

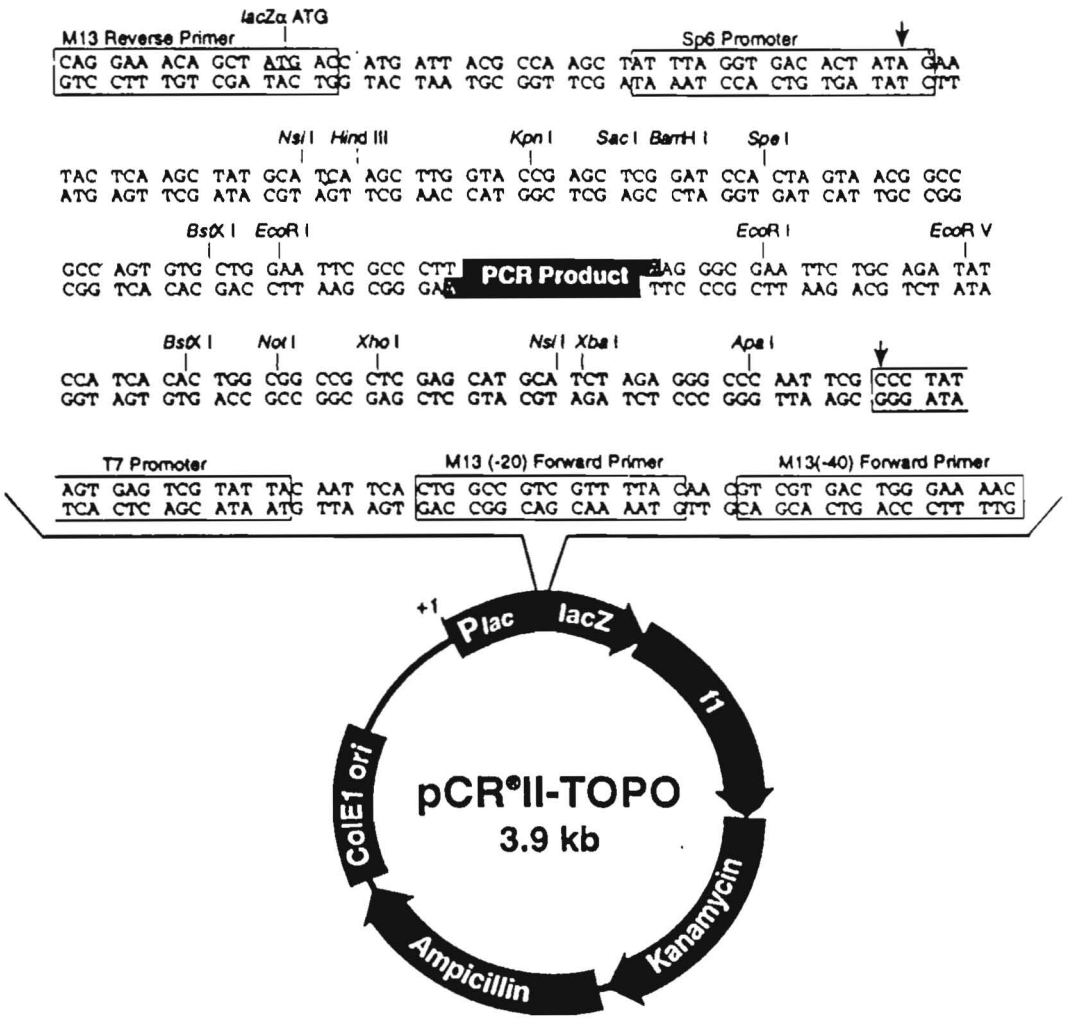
In order to clone the gene into E. coli, a TOPO TA Cloning® kit (Invitrogen Corp., Carlsbad, CA) was used. This cloning kit provided a one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR

products into a plasmid vector. *Taq* polymerase adds a single deoxyadenosine to the 3' ends of PCR products. The vector supplied in this kit (pCR®II-TOPO) has single, overhanging 3' deoxythymidine residues (Fig. 7). This allowed PCR inserts to ligate efficiently with the vector. The pCR®II-TOPO vector was activated with topoisomerase to enhance the ligation activity between the vector and the PCR insert.

To initiate the cloning procedure, 2 µl of the purified PCR product was mixed with 2 µl of sterile water (supplied with the kit) and 1 µl of pCR®II-TOPO plasmid in sterile 0.5 ml microfuge tubes. This mixture was incubated at RT for 5 minutes after which the tubes were immediately put on ice.

The supplied competent TOP10F' *E. coli* cells were stored at -70 C in 50 µl aliquots. Immediately prior to use, the competent cells were thawed on ice. After thawing, 2 µl of 0.5 M β-mercaptoethanol and 2 µl of the cloning reaction were added to each aliquot of cells and mixed by stirring with a pipette tip. This mixture was placed on ice for 30 minutes and then heat shocked at 42 C for 30 seconds. The tubes were returned to ice for 2 minutes. Supplied SOC medium (0.25 ml) was added to each tube to initiate cell growth and the tubes were horizontally shaken for 30 minutes at 37 C. During this incubation period, plates with Luria

Figure 7. The map of the pCR®II-TOPO vector showing the location of the PCR insert.


lacZα ATG
M13 Reverse Primer
Sp6 Promoter
CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TAT TTA GGT GAC ACT ATA GAA
GTC CTT TGT CGA TAC TGG TAC TAA TGC GGT TCG ATA AAT CCA CTG TGA TAT CTT
NsiI HindIII KpnI SacI BamHI SpeI
TAC TCA AGC TAT GCA TCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GCC
ATG AGT TCG ATA CGT AGT TCG AAC CAT GGC TCG AGC CTA GGT GAT CAT TGC CGG
BstXI EcoRI EcoRI EcoRV
GCC AGT GTG CTG GAA TTC GCC CTT PCR Product AAG GGC GAA TTC TGC AGA TAT
CGG TCA CAC GAC CTT AAG CGG GAA TTC CCG CTT AAG ACG TCT ATA
BstXI NotI XhoI NsiI XbaI ApaI
CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
T7 Promoter
M13 (-20) Forward Primer
M13(-40) Forward Primer
AGT GAG TCG TAT TAC AAT TCA CTG GCC GTC GTT TTA CAA CGT CGT GAC TGG GAA AAC
TCA CTC AGC ATA ATG TTA AGT GAC CGG CAG CAA AAT GTT GCA GCA CTG ACC CTT TTG
+1
Plac
lacZ
f1
pCR®II-TOPO
3.9 kb
ColE1 ori
Ampicillin
Kanamycin

Bertani (LB) medium containing 50 µg / ml carbinicillin, (designated as LB-cb50), were prewarmed at RT. The medium surface was treated with 30 µl of X-gal and 50 µl of IPTG, both required for blue/white screening. Following the 30 minute incubation at 37 C, 50 µl of the plasmid/cell suspension was spread over the surface of the plate. Plates were sealed with parafilm and incubated at 37 C overnight. The following morning, selected colonies from each isolate were streaked onto a LB-cb50 replicate plate with a sterile toothpick. The toothpick was immediately dropped into a tube containing 3 ml of LB-cb50 broth. The replicate plate was incubated at 37 C overnight and placed at 5 C. Sealed tubes were placed in a horizontal shaker at 200 rpm at 37 C overnight.

Preparation of plasmid DNA for enzyme digestion

After cells from selected colonies incubated at 37 C overnight, 1.5 ml of the selected cell suspensions were transferred to 1.5 ml microfuge tubes. Cells were pelleted by centrifugation at 16,000 x *g* for 1 minute at 5 C. The supernatant was decanted and the cell pellet resuspended in 0.1 ml of STET. Lysozyme (4 µl of 10 mg / ml STET) was added to each tube. The tubes were placed in boiling water for 1 minute to disrupt the cells thus releasing plasmid DNA into solution. After boiling, the lysed cell debris was

collected by centrifugation at 16,000 x *g* for 10 minutes at 5 C. Following centrifugation, the pellet was removed from the tube with a sterile toothpick and discarded leaving plasmid DNA in the supernatant. Freezer-cold isopropanol (0.1 ml) was added to each tube and mixed well. DNA was collected by centrifugation at 16,000 x *g* for 5 minutes at 5 C. The supernatant was discarded and the pellet resuspended in 0.5 ml freezer-cold 75% ethanol. After centrifugation (16,000 x *g* for 5 minutes at 5 C) the tubes were inverted and allowed to air-dry for 5 minutes. The final plasmid DNA pellet was resuspended in 25 µl of TE buffer.

Enzyme digestion of plasmid

In order to ascertain the incorporation of the insert DNA into the plasmid DNA, an enzyme digestion was necessary to isolate the insert from the plasmid. This procedure was accomplished by digesting the putatively recombinant plasmid with EcoR1. The pCR®II-TOPO plasmid was designed with EcoR1 restriction sites at either side of the PCR product insert site.

The plasmid DNA, (2 µl), was added to 13 µl of an enzyme digestion cocktail comprised of buffer H (1.5 µl), 100x BSA (0.3 µl), EcoR1 (1 µl), and sterile $_{dd}H_2O$ (10.2 µl). The volumes shown are for individual digestion reactions.

The volumes for the enzyme digestion cocktail were increased by the number of individual digestions performed. Digestion reactions were at 37 C for 2 hours and were stopped by the addition of 3 µl of bromophenol blue loading buffer. Digestion results were analyzed by electrophoresis on a 1% agarose gel. To prevent smearing of any restriction fragments, the gel was subjected to low voltage, (25 volts for 15 minutes and then increased to 40 volts for the remainder of the run). The size of restriction fragments was determined by comparing the position of the band in the gel with the position of the bands of the 1 kb DNA ladder marker.

Plasmid purification

Before the insert in the plasmid can be used for sequencing, the plasmid DNA must be purified. Plasmid DNA was purified using a QIAGEN Plasmid Midi kit (QIAGEN Inc., Valencia, CA 91355). This procedure required 3 days to complete. TOP10F' *E. coli* cells, shown to have been transformed with the NIa gene, were transferred from the replicate plate (stored at 5 C) to a sterile 250 ml flask containing 25 ml of LB-cb50 broth. Cells were incubated at 37 C overnight and shaken at 200 rpm. The next day, an additional 25 ml of LB-cb50 broth was added to further increase cell growth and flasks were returned to the

horizontal shaker (same conditions as before) for an additional 24 hours. The TOP10F' cells were collected by centrifuging the cell suspension at 6,000 x *g* for 15 minutes at 5 C. The centrifuge tubes were inverted to remove all traces of the medium from the cell pellet. Each pellet was resuspended in 4 ml of chilled (5 C) Buffer P1 (supplied in kit). After resuspension, cells were lysed by the addition of 4 ml of Buffer P2 (supplied in kit). This mixture was gently mixed by inverting tubes several times and allowed to incubate at RT for 5 minutes. Buffer P2 contains a NaOH-SDS solution with RNase A. SDS solubilized the phospholipid and protein components of the cell membrane, leading to cell lysis and release of cell contents. NaOH denatured the chromosomal and plasmid DNAs, as well as proteins while RNase A digested the released RNA. After the 5 minute incubation, 4 ml of chilled (5 C) Buffer P3 (supplied in kit) was added to each tube, gently mixed by inverting tubes several times, and placed on ice for 15 minutes. Plasmid DNA was separated from cell debris and other solution components by centrifugation at 20,000 x *g* for 30 minutes at 5 C. The resulting clear supernatant containing the plasmid DNA was collected and re-centrifuged at 20,000 x *g* for 15 minutes at 5 C. The supernatant was then immediately loaded onto a pre-equilibrated QIAGEN-tip column (supplied with kit). Salt and pH conditions of the column allowed

only plasmid DNA to bind to the QIAGEN Resin while other cellular components did not. The column was washed with 10 ml of Buffer QC (supplied with kit) twice to remove any remaining contaminants. Plasmid DNA was then eluted by the addition of 5 mL of Buffer QF (supplied with kit). The plasmid DNA was then precipitated by the addition of 0.9 volumes (4.5 ml) of freezer-cold isopropanol. Each plasmid DNA/isopropanol mixture was mixed and immediately centrifuged at 15,000 x *g* for 30 minutes at 5 C. Each DNA pellets was resuspended in 0.1 ml of 10 mM Tris-Cl (pH 8.3) and 10 µl of 3 M $NaCH_3COO$ (pH 5.0). Each tube was placed in a -20 C freezer for 1 hour and mixed every 20 minutes. The resuspended DNA pellets were transferred to 1.5 ml microfuge tubes and precipitated by the addition of 2 volumes (0.220 ml) of 75% EtOH. The DNA pellets were collected by centrifugation at 16,000 x *g* for 20 minutes at 5 C. Each purified plasmid DNA pellet was redissolved in 50 µl of 10 mM Tris-Cl, pH 8.5. DNA concentration was determined by UV spectrophotometry. A sample from each virus isolate was sent to the DNA Sequencing Facility at Iowa State University, Ames, IA for nucleotide sequencing of the NIa insert.

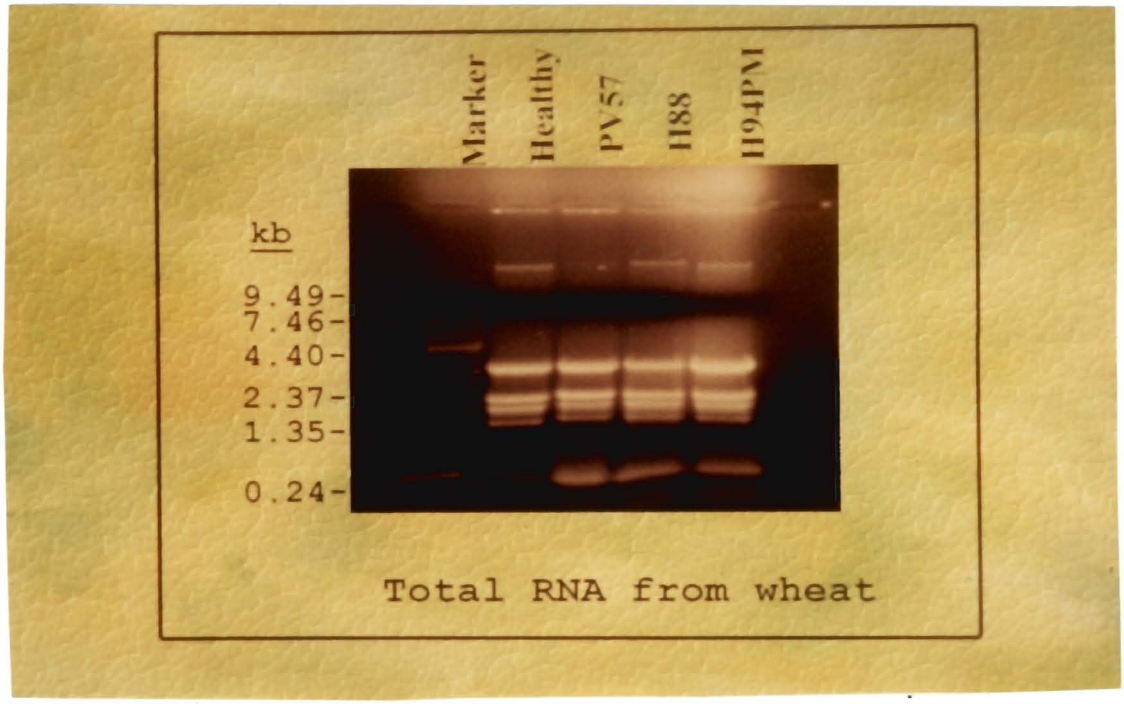
CHAPTER 3

RESULTS

RNA integrity

Total RNA integrity was determined by electrophoresis on a 1% agarose gel (Fig. 8). There was no noticeable difference between RNA extracted from healthy or WSMV-infected wheat and there was no noticeable difference among the different strains of WSMV. The presence of discrete bands indicates intact RNA. There was minimal degradation through the RNA extraction procedure. Degraded RNA caused lanes on a gel to appear as a smear consisting of small RNA fragments (not shown). The majority of the visible bands were ribosomal RNAs ranging from 0.24 to approximately 10 kb although messenger and transfer RNAs were also present in the total RNA sample. Viral RNA was not visible at this step in the procedure due to the inherent low viral concentration of WSMV *in vivo* and the lack of a viral purification procedure. No attempt was made to purify the virus as the specificity of the oligonucleotide primers used during RT-PCR was sufficent to recognize the viral RNA sequence within the total RNA sample. Only RNA samples that did not show a high degree of degradation were used for subsequent steps in the procedure.

Figure 8. A sample of total RNA extracted from both healthy and WSMV-infected wheat and subjected to electrophoresis on a 1% agarose gel.


Marker
Healthy
PV57
1188
1194PVI
kb
9.49-
7.46-
4.40-
2.37-
1.35-
0.24-
Total RNA from wheat

RNA quantification

The amount of total RNA initially extracted was determined by ultraviolet (UV) spectroscopy. RNA quantity (µg / µl) was determined by recording optical density readings at a wavelength of 260 nanometers (OD_{260}) of a 1:100 dilution of the total RNA sample (Table 1). The product of the OD_{260} reading and the dilution factor was multipled by a correction factor of 40. (For single-stranded RNA, an OD_{260} of 1.0 indicates 40 µg / ml). This datum was then converted to µg / µL by dividing by 1000.

There was no noticeable difference of RNA quantity extracted from healthy or WSMV-infected wheat. The lowest average RNA quantity was from wheat infected with the PV57 isolate however this was not noticeably lower. The final RNA quantity for healthy and all WSMV isolates approximated 1 µg / µl. OD_{280} readings were also taken to determine if the aqueous phase containing RNA was contaminated with the phenol phase containing DNA and proteins. Literature from Gibco BRL Life Technologies stated the $OD_{260/280}$ ratio should be greater than 1.65 for RNA samples. $OD_{260/280}$ ratios equal to or less than 1.65 indicate phenol phase contamination in the RNA sample. Data presented in Table 1 show averages of 3 separate trials including the largest deviation from each average. Two RNA samples (healthy and H94PM) had $OD_{260/280}$ ratios equal to and

Table 1. Ultraviolet Absorbance of Total RNA Isolated from Wheat Infected with Wheat Streak Mosaic Virus and Corresponding RNA Concentration

	UV Wavelength			
Isolates	**OD_{260} [a]**	**OD_{280}**	**OD_{260} / OD_{280}**	**µg RNA / µL [b]**
Healthy	0.233 ± 0.08	0.141 ± 0.04	1.65	0.932
WSMV-PV57	0.229 ± 0.08	0.134 ± 0.04	1.71	0.916
WSMV-H88	0.261 ± 0.09	0.152 ± 0.03	1.71	1.044
WSMV-H94PM	0.256 ± 0.05	0.158 ± 0.04	1.62	1.024

[a] = optical density (OD) readings are from a 1:100 dilution of final RNA preparation. Averages of 3 trials are reported with the largest deviation reported for each average.

[b] = RNA concentration is determined by: [(OD_{260} x dilution factor x 40) / 1000]

slightly less than 1.65, respectively. All samples were used for subsequent procedures although there may have been some phenol phase contamination as indicated by the $OD_{260/280}$ ratios.

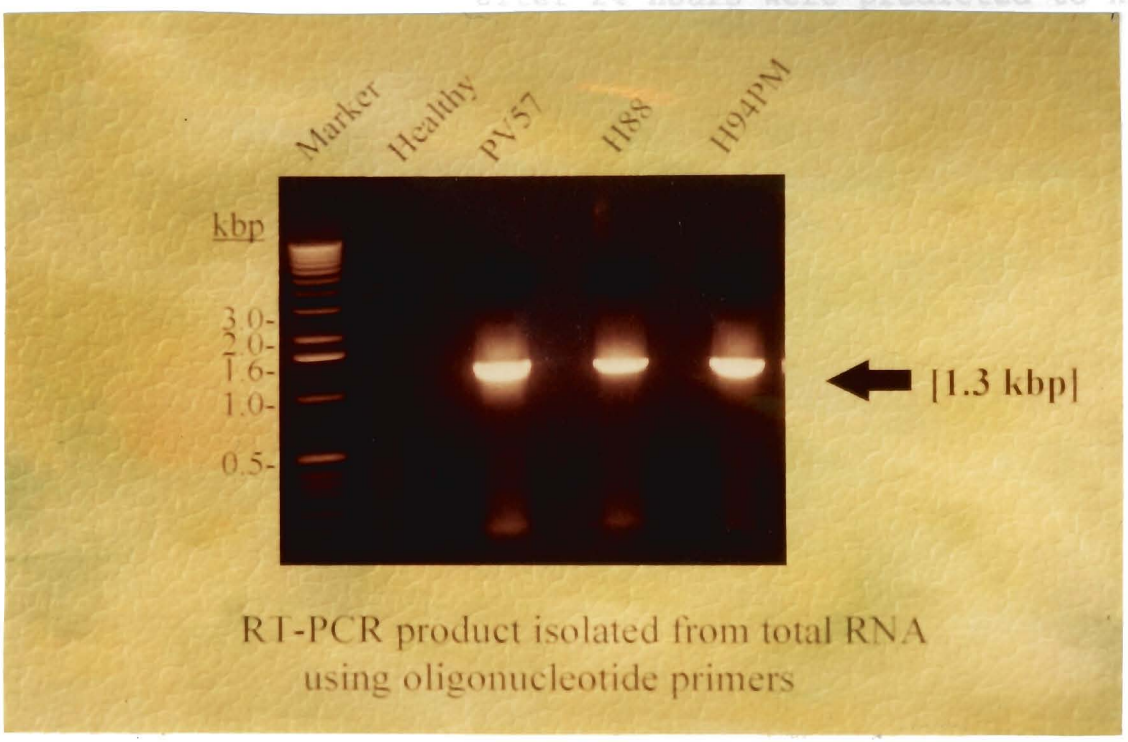
RT-PCR of the NIa gene

Before the NIa sequence could be amplified by PCR, complementary DNA (cDNA) had to be synthesized by reverse transcription from the single-stranded RNA template. A sample of the RT-PCR product was analyzed on a 1% agarose gel (Fig. 9). The specificity of the oligonucleotide primers was shown by the presence of a band in the WSMV-infected lanes and the absence of any bands in the healthy control lane. Comparison of the visible band with the DNA marker indicated an approximate size of 1.3 kilobasepair (kbp).

The amplified cDNA gene was eluted from the agarose gel by using the QIAEX II kit as described in the Material and Methods section and quantified on an agarose gel by comparing the band intensity with the intensity of bands on a DNA Low Mass Ladder. Final cDNA concentrations were approximately 10 ng / µl (data not shown).

Figure 9. A sample of the product of RT-PCR subjected to electrophoresis on a 1% agarose gel for healthy, PV57, H88, and H94PM infected wheat.

after 24 hours were predicted to have



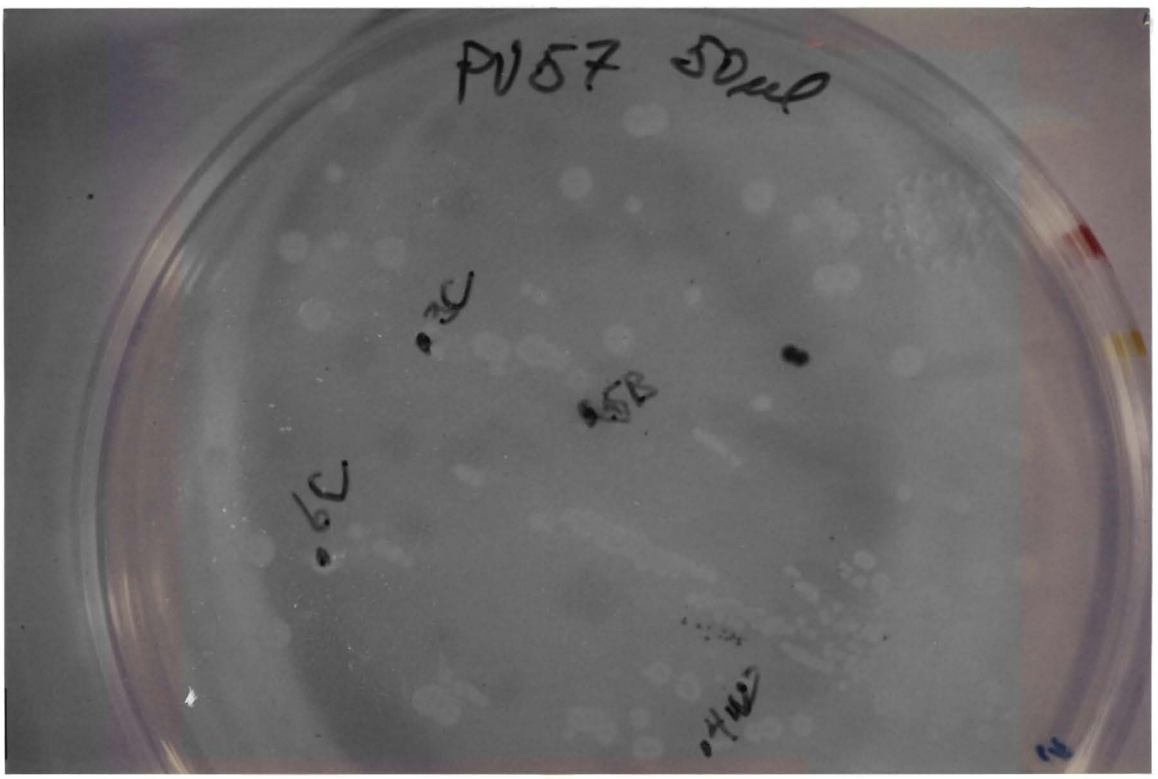
RT-PCR product isolated from total RNA using oligonucleotide primers

Cloning of NIa gene into *E. coli*

The purified NIa DNA was successfully cloned into TOP10F' *E. coli* cells by using a TOPO TA Cloning® kit which included the pCR®II-TOPO plasmid vector. Transformed *E. coli* cells were spread onto LB-cb50 media treated with IPTG and X-gal and incubated at 37 C for 24 hours. Off-white colonies present after 24 hours were predicted to have incorporated the PCR insert while blue colonies were predicted to not include the PCR insert (Fig. 10). Colonies seen in fugure 10 were representative of colonies ligated with the NIa gene from the other WSMV isolates. Several colonies exhibited a brighter white color. These colonies were also predicted to not include the PCR insert. The pCR®II-TOPO plasmid, which was pre-digested at the PCR product insert site, apparently recombined allowing the ampicillin-resistant gene to function and allowed for growth on the selective medium even though the PCR product insert was not incorporated in the case of the bright white and blue colonies.

Selected colonies of the three phenotypes for each virus isolate were aseptically transferred to a replicate LB-cb50 plate for storage at 5 C (Fig. 11).

Figure 10. LB-cb50 media inoculated with *E. coli* cells transformed with NIa inserts from WSMV-PV57.


PV57 50ml
3C
5B
6C

EcoR1 digestion of plasmids

The NIa insert was extracted from the transformed TOP10F' *E. coli* cells by digestion with the EcoR1 enzyme. EcoR1 was a very useful restriction enzyme for this vector as there was an EcoR1 restriction site on either side of the PCR product insert site. Figure 11 shows the presence of an approximate 1.3 kbp DNA fragment from cells originating from colonies with the off-white color. H88 and H94PM PCR appear to have an EcoR1 restriction site within the NIa gene sequence as the sum of the two band sizes equals 1.3 kbp while the insert extracted from cells transformed with the PV57 PCR product appears as a single 1.3 kbp band.

Cells from the blue and bright white colonies from all three WSMV isolates did not contain the PCR product insert. A sample of cells shown to have been transformed with the NIa gene were prepared for long-term storage by using them to inoculate 1.5 ml microfuge tubes with LB-cb50 broth mixed with glycerol and stored at -20 C.

Nucleotide sequence analysis of the NIa gene

Samples of purified PCR products and purified plasmid DNA of the three virus strains were sent to the DNA Sequencing Facilities at Iowa State University along with samples of the custom primers. Nucleotide sequencing of the PCR products employing identical primers as during RT-PCR

Figure 11. Selected colonies from transformation experiment transferred to replicate plate for cold storage.

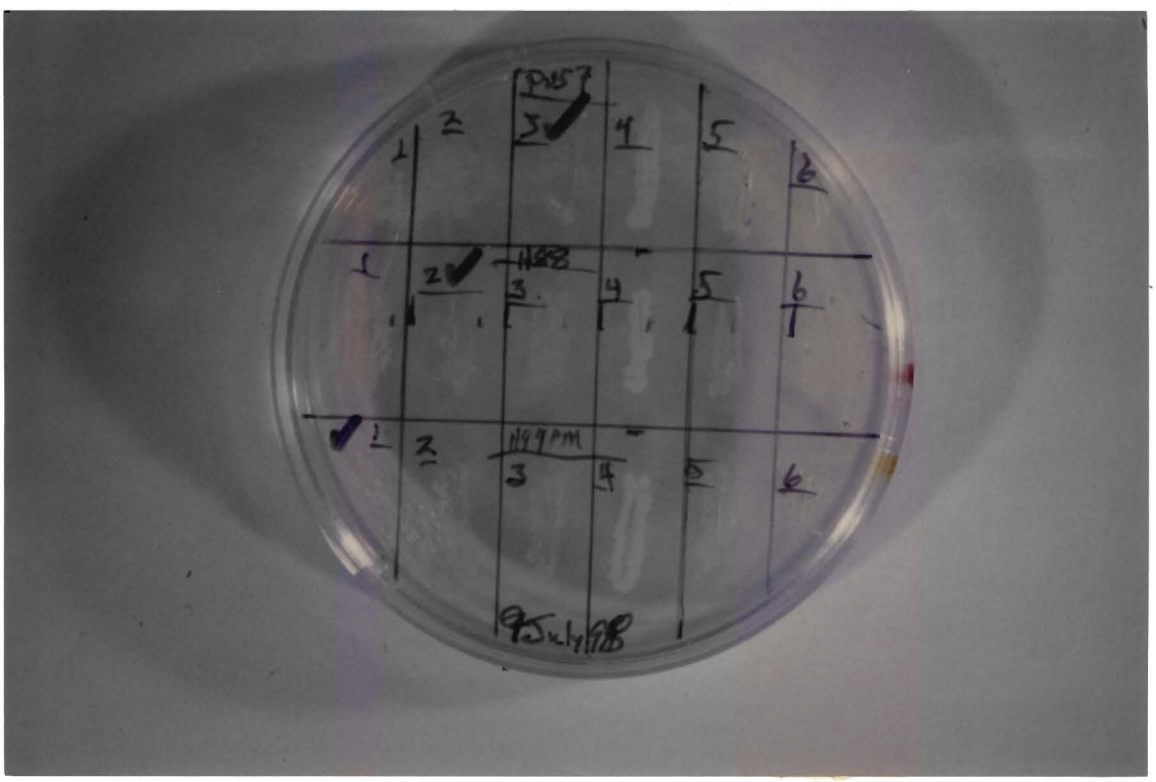
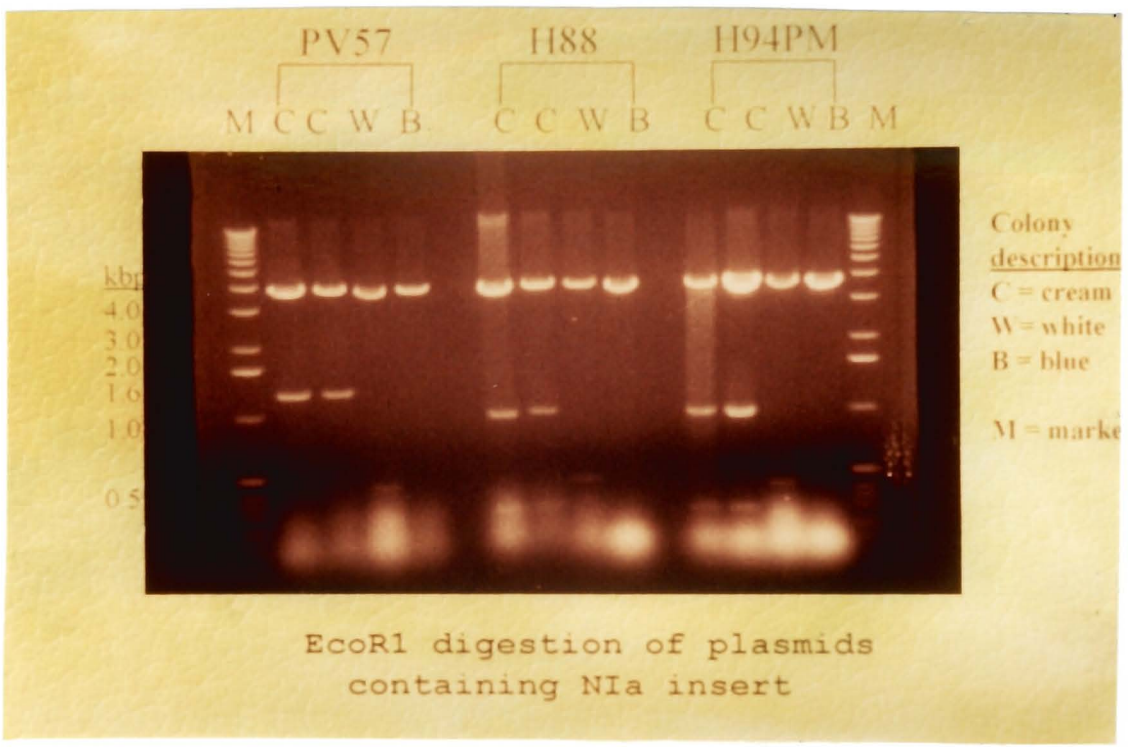

P45
1
2
3
4
5
6
H88
1
2
3
4
5
6
H89PM
1
2
3
4
5
6

Figure 12. EcoR1 digestion of *E. coli* cells transformed with the NIa inserts.



EcoR1 digestion of plasmids containing NIa insert

was unsuccessful. Reliable sequencing continued for approximately 300-400 bases from each primer before sequence degradation occurred making the reported sequence unreliable (data not shown). This left much of the sequence between each primer unknown as the reported length of the NIa gene is approximately 1.3 kb.

The complete nucleotide sequence of the Nia gene was successfully determined from purified plasmid DNA (Table 2). Nucleotide sequences originating from T7 and Sp6 promoter sites within the pCRII®-TOPO plasmid genome were overlapped with nucleotide sequences from custom primers which basepaired with nucleotides towards the middle of the NIa gene. This combination supplied sufficient data to determine the nucleotide sequence of the entire length of the NIa gene. The nucleotide sequences were screened for most probable identity by using two on-line genetic data banks: **[http://dot.imgen.bcm.tmc.edu]** and **[http://www.ncbi.nlm.nih.gov]**. All sequences from each primer were most similar with a section of a sequence of WSMV-Sidney 81 which has its complete genome sequence on-line.

Nucleotide sequence alignment showed significant similarity among the three WSMV strains. The three NIa nucleotide sequences were 97.3 % similar. Within the 1268

base sequence of the NIa gene, there were 2 sites of ambiguity, resulting in an unknown base at each site. This ambiguity (represented by an "n") occurred in each isolate once. PV57 and H94PM had an "n" at base #433 while H88 had an "n" at base #910.

Of the 34 sites of different nucleotide sequence, H88 and H94PM were similar at 20 sites, PV57 and H88 were similar at 6 sites, as were PV57 and H94PM. This resulted in a 98.9% NIa nucleotide sequence similarity between H88 and H94PM, and a 97.8% NIa nucleotide sequence similarity between the PV57 : H88 combination and the PV57 : H94PM combination. Amino acid sequence alignment was beyond the scope of this project, therefore the number of silent differences is unknown.

The sequence shown in figure 12 suggests the NIa gene from the H88 and H94PM strains contains an EcoR1 restriction site while the NIa gene from PV57 does not. Table 2 shows an EcoR1 restriction site (GAATTC) at base #267 for H88 and H94PM that is not present in the NIa gene sequence for PV57.

Table 2. NUCLEOTIDE SEQUENCE ALIGNMENT OF NIa GENE FROM WSMV

```
PV57       1   cacgcaacag gagtgcaaaa cgacaatcag caagagatca aaagatggag cgtggcaacg aatacacata ctacgatgct ggtgacacct
H88            cacgcaacag gagtgcaaaa cgacaatcag caagagacca aaagatggag cgtggcaacg aatatacata ctacgatgct ggtgacacct
H94PM          cacgcaacag gagtgcaaaa cgacaatcag caagagatca aaagatggag cgtggcaacg aatacacgta ctacgatgct ggtgacacct

PV57      91   tgtataatgg agttcaagag aatatgaatc atgcaccaga ctggaccgac cggattaaga agaagactca tgcatacgca atgcaatttg
H88            tgtataatgg agttcaagag aatatgaatc atgcaccaga ctggaccgat cggattaaga agaagactca tgcatacgct atgcaatttg
H94PM          tgtataatgg agttcaagag aatatgaatc atgcaccaga ctggaccgac cggattaaga agaagactca tgcatacgct atgcaatttg

PV57     181   gtagggaagt accaaaaact gaagcacagc gatcctcaca atactggcac ttctacggtt ttgatccaaa gatgtatgat tcagtcgaat
H88            gtagggaagt accaaagact gaaacacagc aatcctcaca atactggcac ttctacggtt ttgatccaaa gatgtatgac tcagtcgaat
H94PM          gtagggaagt accaaagact gaaacacagc gatcctcaca atactggcac ttctacggtt ttgatccaaa gatgtatgac tcagtcgaat

PV57     271   ttaaggacat atcagcaaac ttctcagtgc atcaggatgc aaaggcaatg gatttgcaga aggccttcac agaaatggtg gaaaatcatt
H88            tcaaggacat agcagcaaac ttctcagtgc accaggatgc aaaggcaatg gatttgcaga aagccttcac agaaatggtg gaaaatcgtt
H94PM          tcaaggacat agcagcaaac ttctcagtgc accaggatgc aaaggcaatg gatttgcaga aagccttcac agaaatggtg gaaaatcgtt

PV57     361   gggatgatga agacttcttc gacgagaaga taccaaagcg agttttggcc atcttcagga aaggagacaa ggntcgtgaa gttgcattgg
H88            gggatgatga agacttcttc gacgagaaga taccaaagcg agttttggcc atcttcagga aaggagacaa ggttcgtgaa gttgcattgg
H94PM          gggatgatga agacttcttc gacgagaaga taccaaagcg agttttggcc atcttcagga aaggagacaa ggntcgtgaa gttgcattgg
```

NUCLEOTIDE SEQUENCE ALIGNMENT OF NIa GENE FROM WSMV (p. 2)

```
PV57    451  cacctcacaa gccaaaccaa gtcaacaagc gtgggctacc cgtcggacat gctgatcaca gaggagagtg gagacaaaca cagccttcat
H88          cacctcacaa gccaaaccaa gtcaacaagc gcgggctacc tgtcggacat gctgatcaca gaggagagtg gagacaaaca cagccttcat
H94PM        cacctcacaa gccaaaccaa gtcaacaagc gtgggctacc tgttggacat gctgatcaca gaggagagtg gagacaaaca cagccttcat

PV57    541  ttgaaaaaga agtgtcgtac gagaacaaat caactttcga aggtgcacgt tcacttgatc atatccatca gaatcaagtc atcctcgttg
H88          ttgaaaaaga agtgtcgtac gagaacaaat caactttcga aggtgcacgt tcacttgatt atatccatca gaatcaagtc atcctcgttg
H94PM        ttgaaaaaga agtgtcgtac gagaacaaat caactttcga aggtgcacgt tcacttgatc atatccatca gaatcaagtc atcctcgttg

PV57    631  aagacaatca acagttaaat gggctaatag ttggaaacat actcttggcg ccatatcatt tcacacgggg tatgaggaac agagaggaga
H88          aagacaatca gcagttaaat gggctaatag ttgggaacat actcttggcg ccatatcatt tcacacgagg tatgaggaac agagaggaga
H94PM        aagacaatca gcagttaaat gggctaatag ttggaaacat actcttggcg ccatatcatt tcacacgagg tatgaggaac agagaggaga

PV57    721  aggaaacacg catgttgaca cagtttggaa cgtataatct tggaaaactt accaacaagc atgtcacaaa attcacaatg atggatctgg
H88          aggagacacg catgttgaca cagtttggaa cgtacaatct tggaaaactt accaacaagc atgtcacaaa atttacaatg atggatctgg
H94PM        aagagacacg catgttgaca cagtttggaa cgtacaatct tggaaaactt accaacaagc atgtcacaaa atttacaatg atggatctgg

PV57    811  tagcattaac attacctcca acatttcaag caagacggaa actcaaatgt ttcagaccac caagggaagg agagcgagca atgctggtga
H88          tagcattaac gttgcctcca acatttcaag caagacggaa actcaaatgt ttcagaccac caagggaggg agagcgagca atgttggtga
H94PM        tagcattaac gttgcctcca acatttcaag cgagacggaa actcaaatgt ttcagaccac caagggaagg agagcgagca atgttggtga
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NUCLEOTIDE SEQUENCE ALIGNMENT OF NIa GENE FROM WSMV (p.3)

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PV57   901    ccatgcagta cgagaaagca ggatgggttg ccaagcaatc agcggaaaca acaatcacac catttggtga tcgacatgat ggtttgtgga
H88            ccatgcagtn cgagaaagca ggatgggttg ccaagcaatc agcagaaaca acaatcacac cattcggtga tcgacatgat ggtttgtgga
H94PM          ccatgcagta cgagaaagca gggtgggttg ccaagcaatc agcagaaaca acaatcacac cattcggtga tcgacatgat ggtttgtgga

PV57   991    agcatagaat ttcaacagga ccaggtgact gtggaagtgc catagtagca gtagcagacc taaaagttgt gggattccat aaccttggag
H88            agcatagaat ttcaacagga ccaggtgact gtggaagcgc catagtagca gtagcagacc taaaagttgt gggattccat aaccttggag
H94PM          agcatagaat gtcaacagga ccaggtgact gtggaagcgc catagtagca gtagcagacc taaaagttgt gggattccat aatcttggag

PV57   1081   ggaaaggtga gaattacttc acaccgataa ctattgaggt catggatttc ttagctgaaa agtctgtaac accgcttgtg ccatggaagt
H88            ggaaaggtga gaattatttc acaccgataa ctattgaggt catggatttc ttagctgaaa aacctgtgac accgcttgtg ccatggaagt
H94PM          ggaaaggtga gaattacttc acaccgataa ctattgaggt catggatttc ttagctgaaa agtctgtgac accgcttgtg ccatggaagt

PV57   1171   tctcagacga gcaagttgac ttgtgtggtt taattgcgac caatggagca gacaaatacc cattcactaa aacaacaagc gacttggtta
H88            tctcagacga gcaagttgac ttatgtggtt taattgcggc caatggagca gacaaatacc cattcaccaa aacaataagc gacttggtta
H94PM          tctcagacga gcaagttgac ttatgtggtt taattgcggc caatggagca gacaaatacc cattcaccaa aacaataagc gacttggtta

PV57   1261   gttggcaa
H88            gttggcaa
H94PM          gttggcaa
```

CHAPTER 4

DISCUSSION

Total RNA vs viral RNA

Wheat cells infected with WSMV have several different types of RNA present. When total RNA was extracted from crude plant extracts, the NIa protease gene, which is part of the WSMV genome, was assumed to be represented in the RNA extraction, along with ribosomal, messenger and transfer RNAs. Healthy and WSMV-infected samples of the initial RNA extraction, when subjected to electrophoresis on an agarose gel, showed no difference in the positions of RNA bands (Fig. 8). This was not unexpected as the concentration of viral RNA was observed to be approximately two orders of magnitude less than the concentration of total RNA and would not be visible as a separate band on an agarose gel without being purified. This comparison is based on the concentration of total RNA from the initial RNA extraction (1 μg / μl) and the concentration of the purified NIa gene (10 ng / μl). These data were obtained by quantifying samples of RNA and cDNA and would not be valid to predict actual amounts of RNA *in vivo*. As the WSMV virions were not purified, the actual contribution of WSMV-RNA to total RNA is unknown although Dougherty (5) reported TEV-RNA contributes as little as 0.0001% to total RNA.

RT-PCR of the NIa gene

Before the NIa gene could be amplified by PCR, cDNA was synthesized by reverse transcription. The NIa template is part of the potyviral genome and was assumed to be in the total RNA preparation. cDNA synthesis was performed with reverse transcriptase isolated from avian mylian virus (AMV). Literature from Boehringer Mannheim suggested cDNA synthesis should be performed for 30 minutes at 42 C if *Taq* DNA polymerase would be used for subsequent cDNA amplification (PCR) although the cDNA synthesis was performed at 50 C for 30 minutes successfully (Fig. 9). An advantage of the Titan™ One Tube RT-PCR System was that the reverse transcriptase and *Taq* DNA polymerase could be combined in the same tube, eliminating any need to disturb the reaction once it was placed in the thermal cycler thus reducing the chance of contamination.

By utilizing custom-made oligonucleotide primers, which base-pair to and define each end of the NIa gene, the action of the *Taq* DNA polymerase resulted in pieces of DNA that accumulated geometrically. Even if the PCR started with only one molecule of cDNA, after 35 cycles, the number of amplified molecules of DNA would theoretically exceed 34 billion (2^{35}). The amplified DNA was of the same size as shown in Figure 9. The absence of a band in the healthy

wheat lane showed that the oligonucleotide primers did not recognize a base sequence to base-pair with in the cDNA preparation. This suggests that the NIa gene is not part of the healthy wheat genome.

Cloning of the NIa gene

Competent TOP10F' *E. coli* cells were transformed with the NIa gene by using the TOPO TA Cloning® kit which included the pCR®II-TOPO plasmid vector. This cloning kit was designed for efficient ligation of PCR products amplified by the *Taq* DNA polymerase. This was accomplished due to the fact that *Taq* polymerase has a terminal transferase activity which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The pCR®II-TOPO plasmid vector is supplied linearized with single overhanging 3'deoxythymidine (T) residues. Ligation of the vector with the *Taq* polymerase-amplified PCR insert occurred spontaneously within 5 minutes at RT.

Selective medium (LB-cb50) was prepared with the addition of the antibiotic, carbinacillin, as opposed to ampicillin. The amp_r gene located in the genome of the pCR®II-TOPO plasmid vector also functions to induce resistance to carbinacillin. It was common lab practice at KSU to use carbinacillin in place of ampicillin because it

had been observed that carbinacillin remained active for longer periods of time.

The success of the transformation was evident by the number of off-white, cream-colored TOP10F' *E. coli* colonies present on LB-cb50 media after 24 hours. The presence of carbinacillin in the LB-cb50 medium killed most of the cells. Cells that acquired the plasmid containing the NIa insert were resistant to carbinacillin because of the amp_r gene.

Because the plasmid came prelinearized, the ligation site of the gene insert was between the lac promoter (P*lac*) and the *lacZ* gene which produces β-galactosidase which serves to cleave the X-gal substrate, producing a blue color. TOP10F' cells normally have a repressor (laclq) on the lac promoter. IPTG binds with the laclq repressor so that it cannot bind with the lac promoter. This allows the lac promoter to be on which allows the inserted gene product to be expressed. The NIa gene insert was ligated in between Plac and lacZ, causing lacZ to be terminated, resulting in no β-galactosidase activity. This caused the formation of white TOP10F' cells. The blue TOP10F' colonies were colonies with a functional *lacZ* gene product (β-galactosidase) which cleaved the X-gal substrate, producing a blue color. The bright-white colonies were

thought to be contaminants.

Random colonies were selected for subsequent EcoR1 enzyme digestion. Blue and bright-white colonies were selected for digestion even though literature supplied with the cloning kit noted that blue colonies would not contain the PCR insert.

Successful cloning of the NIa gene makes long-term storage of a purified gene possible and also supplies future investigators with easy access to the NIa gene. This will prove invaluble when investigators begin the process of engineering primary, secondary, and tertiary structures of an inhibitor gene that will block the cleavage activity of the NIa gene.

EcoR1 digestion of plasmid DNA

Examination of the plasmid genetic map (Fig. 7) shows EcoR1 restriction sites (GAATTC) immediately at both ends of the PCR product insert site. This design made EcoR1 an obvious choice to use during the enzyme digest. After a 2 hour digest, figure 12 shows the presence of a restriction fragment removed from plamid DNA from cream-colored colonies that approximates the predicted size of 1.3 kbp for the NIa gene. Blue and white colonies did not contain the PCR insert as predicted. Close examination of lanes containing H88 and H94PM inserts showed two restriction

fragments with sizes that add to 1.3 kbp. This suggests there are EcoR1 restriction sites within the NIa gene sequence of these two WSMV isolates. Table 2 shows the nucleotide sequence alignment of the NIa gene for each of the three WSMV strains in this study. At base #267, a 'GAATTC' sequence is present in the H88 and H94PM sequence but is not found in the PV57 NIa sequence. This helps to explain the band positions in figure 12. With an EcoR1 restriction site at base #267, there should be two bands (1000 bp and 260 bp) present in H88 and H94PM.

This nucleotide sequence difference is an example of biological variation of the NIa gene. The two WSMV isolates with the EcoR1 restriction site were isolated at Hays, KS, while PV57 is considered to be the type specimen of WSMV. There is also biological variation among the three strains with regards to host reservoirs. H94PM was isolated from pearl millet whereas H88 and PV57 are unable to infect pearl millet. It is unknown at this time whether NIa activity plays any role in host specificity for WSMV.

Sequence analysis of the NIa gene

Primers used during RT-PCR were designed to recognize nucleotide sequence sites at both ends of the NIa gene. However, these primers, when used for determination of nucleotide sequence, yielded less than optimal results.

Reliable sequence lasted approximately 200-300 bases from each primer, leaving much of the sequence between the primers unknown. During phone conversations with technicians at the DNA sequencing facility at Iowa State University, it was learned that occasionally oligonucleotide primers that worked for the PCR do not work in the sequencing reaction. No further explanation was offered by the technicians other than to try again.

Reliable nucleotide sequence for the complete NIa gene was obtained when the NIa gene was sent for sequencing ligated in pCR®II-TOPO plasmid DNA. The genetic map of the pCR®II-TOPO plasmid DNA (Figure 7) shows a "T7" promoter site upstream and an "Sp6" promoter site downstream from the insertion site of the NIa gene. The DNA Sequencing Facility at ISU maintained primers that recognized these promoter sites in the pCR®II-TOPO plasmid DNA. Each primer initiated nucleotide sequence in opposite directions (upstream and downstream from the NIa gene) and yielded positive sequence results for approximately 600-700 bases in either direction. A custom-designed oligonucleotide primer that will base-pair with the sequence in the middle of the NIa gene was also used as a midway primer.

Each nucleotide sequence was identified by base similiarity using two on-line sequence databases:

[http://dot.imgen.bcm.tmc.edu] and [http://www.ncbi.nlm.nih.gov]. The sequence from each primer for all WSMV strains was most compatible with WSMV-Sidney 81 genome starting at base #5424. Experimental sequences were compared with the published WSMV sequence in order to determine the length and position of nucleotide overlaps.

The nucleotide sequence initiating from the T7 promoter site was the "plus" strand of DNA while the sequence initiating from the Sp6 promoter site was the reverse complement of the "plus" strand. The nucleotide sequence initiating from the custom-designed primer was also the "plus" strand of DNA. Sequences from the T7 and Sp6 promoter sites overlapped with the sequence from the middle primer and provided sufficient data to determine the nucleotide sequence of the complete NIa gene for each WSMV isolate.

Nucleotide sequence alignment of all isolates showed an overall 97.3% base similarity. One notable difference was the presence of an EcoR1 restriction site in the NIa gene sequence found in H88 and H94PM although this did not affect the functionality of the NIa gene. The ubiquity of the sequence should increase the probability that a genetically-engineered inhibitor gene would be effective on several

different isolates of WSMV. The number of different WSMV strains in nature is unknown. This study focused on only three WSMV strains. Additional effort would have to be invested before there could be a high degree of confidence that NIa genes from all WSMV isolates have similar nucleotide sequences.

CHAPTER 5

SUMMARY

Wheat streak mosaic virus is an economically significant pathogen of wheat in Kansas. Over the last 22 years, WSMV has been responsible for a 2.01% average reduction in yield statewide. Wheat breeders are constantly looking for lines of wheat that offer natural resistance to WSMV. Resistance to WSMV could also be achieved if the mode of virion replication was disrupted. This project is the beginning of a larger project that will attempt to develop a method of disrupting the virion replication cycle by genetically engineering a gene that will inhibit the polyprotein cleavage action of protease genes such as NIa. The NIa protease gene is one of several replicase genes found in potyviruses that cleave the polyprotein at certain sites which then allows for viral replication.

Before such a protease inhibitor gene can be genetically engineered, basic information about the genetics of the NIa gene in WSMV must be obtained. Three different strains of WSMV were used in this project. The NIa protease gene from each strain was isolated and amplified using PCR. The NIa protease gene from each virus strain was cloned in TOP10F' *E. coli* cells by ligation of the NIa protease gene

into pCR®II-TOPO plasmid DNA. Nucleotide sequences of each NIa protease gene were obtained by sending purified pCR®II-TOPO plasmid DNA to the DNA Sequencing Facility at Iowa State University. Attempts to sequence the PCR product were unsuccessful. Returned nucleotide sequences were screened for most probable identity using on-line genetic screening sites. All returned nucleotide sequences matched some portions of the complete WSMV-Sidney 81 genome. Significant nucleotide sequence similarity of the NIa protease genes from the three different WSMV isolates was determined by assessing the nucleotide sequence alignment. Of the 2.7% nucleotide differences among the three nucleotide sequences, it is unknown how many are silent differences. This will be determined by performing an amino acid sequence alignment with the three NIa sequences. It is predicted that the amino acid sequence difference will be less than the nucleotide sequence difference making the three NIa genes even more genetically similar.

It is hoped that these findings will allow a NIa protease inhibitor to function on all isolates of WSMV, although this is by no means an all inclusive study of nucleotide sequence similarity of NIa genes from all WSMV isolates. It is also unknown as to the degree of nucleotide similarity necessary for a NIa protease inhibitor to

recognize and bind to the NIa protease gene.

CHAPTER 6

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APPENDIX

STOCK SOLUTIONS OF BUFFERS AND MEDIA

DEPC-treated water

2 mL diethyl pyrocarbamate per liter of H_2O

Let stand for 24 hours and then autoclaved

ethidium bromide (10 mg / mL)

Dissolve 0.2 g ethidium bromide in 20 mL of H_2O

Mix well and store at 5 C in dark

IPTG (100 mM)

Dissolve 238 mg of IPTG in 10 mL H_2O

Filter-sterilize and store in 1 mL aliquots at -20 C

(Do not autoclave)

loading buffer for RNA gels

5 mL glycerol

20 mg bromophenol blue

20 mg xylene cyanol

Mix and add H_2O to 10 mL

loading buffer for DNA gels

18 mL H_2O

50 mg xylene cyanol

1.25 mL 0.50 M EDTA

3.75 g Ficoll (Type 400-DL)

Mix and add H_2O to 25 mL

Filter-sterilize

Luria-Bertani (LB) Medium (pH 7.0)

1.0% Tryptone

0.5% Yeast Extract

1.0% NaCl

Mix and dissolve in 950 mL water

Adjust to pH 7.0 with NaOH and bring volume to 1 liter

Autoclave for 20 minutes at 15 psi

Luria-Bertani (LB) Agar Plates

Prepare LB medium as above

Add 15 g agar / liter

Autoclave for 20 minutes at 15 psi

Cool to 55 C and add 0.050 mg carbinicillin (50 μg/mL)

Pour into 10 cm plates

MOPS buffer (10x)

50 mM sodium acetate

10 mM EDTA

Dissolve 41.56 g MOPS in 800 mL H_2O

Mix and add H_2O to 1 liter

SOC Medium

2% tryptone

0.5% yeast extract

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

20 mM glucose

Mix and add H_2O to desired volume

Autoclave for 20 minutes at 15 psi

sodium acetate (3 M)

Dissolve 408 g sodium acetate · 3 H_2O in 800 mL H_2O

Add H_2O to 1 liter

Adjust pH to 5.2 with 3 M acetic acid

STET buffer

8% sucrose

0.5% Triton X-100

50 mM Tris-Cl, pH 8.0

50 mM EDTA

TAE electrophoresis buffer (50x)

242 g Tris base

57.1 mL glacial acetic acid

37.2 g $Na_2EDTA \cdot 2H_2O$

Dissolve solutes in 800 mL H_2O

pH approximately 8.5

TE buffer (pH 8.0)

10 mM Tris · Cl, pH 8.0

1 mM EDTA, pH 8.0

Tris-Cl buffer (10 mM, pH 8.5)

Dissolve 0.121 g Tris base in 800 mL H_2O

Adjust to desired pH with concentrated HCl

Mix and add H_2O to 1 liter

X-Gal (40 mg / mL)

Dissolve 400 mg X-Gal in 10 mL dimethylformamide

Store in brown bottle at -20 C

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David A Karr

Signature of Author

1 November 1998

Date

Isolation of the NIa protease gene in wheat infected with wheat streak mosaic virus

Title of Thesis Project

Doug Cooper

Signature of Graduate Office Staff

11-24-98

Date Received