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Genetic variability of *Hosta virus X* in hosta

A Thesis
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Degree
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Oluseyi Lydia Fajolu
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Dedication

This dissertation is dedicated to the glory of almighty God, who inspires me in every step of my life. It is also dedicated to the love of my life Olufemi Nelson Fajolu who challenges me to seek a better life for our family.

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Abstract

RNA viruses have high potential for genetic variation. This intrinsic variability is due to lack of proof-reading activity of RNA-dependant RNA-polymerase; hence replication of RNA viruses leads to the generation of individuals that differ genetically from their parental viruses. These individuals are referred to as variants or mutants. The dynamic distribution of mutants that compose replicating RNA viruses is termed quasi-species. Through quasi-species, RNA viruses refine their genetic structure and adapt to changing environment especially when moved from one host to the next. Genetic variability of RNA viruses may influence viral diagnosis and the durability of plant resistance to viral infection. Genetic variation leads to emergence of new viruses as well. New human, animal and plant viruses emerge through mutation, recombination and reassortment. Heterogeneity of plant RNA viruses is being studied to establish their evolutionary history and most importantly to design strategies for effective diagnosis and control of viral diseases. This research was aimed at studying the genetic variability of *Hosta virus X* (HVX) isolates using the coat protein (CP) gene and triple gene block 1 (TGB1). Thirty HVX isolates from naturally infected hosta cultivars were obtained from different geographical regions of Tennessee. These isolates, along with previously reported HVX isolates, and representative species of *Flexiviridae* family were used to infer phylogenetic relationships. The widespread presence of HVX in the State of Tennessee was established. Based on comparison of sequences of CP and TGB1, it was demonstrated that all HVX isolates sequenced to date are closely related, and that the CP gene is more conserved than TGB1 among the isolates of the virus. Based on these

findings, a reverse transcription–polymerase chain reaction combined with restriction fragment length polymorphism assay (RTLP) assay was developed for sensitive detection of HVX in hosta.

Table of Contents

Lists of Tables

Lists of Figures

CHAPTER 1: Introduction

Plant pathogens are responsible for significant annual losses of agricultural products. In the United States of America (USA), approximately $33 billion annual crop losses are due to plant pathogens (50). Plant viruses cause a significant amount of these losses and are second only to fungi among the pathogens that cause economically important losses in agriculture (12, 16). There are over 70 plant viral genera (11) and RNA viruses constitute the largest fraction (43). RNA viruses utilize various strategies to ensure their continuity within their host, escape control measures and expand their host range (15).

1.1 RNA viruses

RNA viruses have ribonucleic acid (RNA) as their genetic material and are classified into four distinct groups. Positive-sense single-stranded RNA viruses (ssRNA) have a genome that acts as mRNA upon infection and can be immediately translated. Negative-sense ssRNA viruses have a genome complementary to mRNA and must be converted to positive-sense RNA before translation. Reverse transcribing RNA viruses (retroviruses), contain a reverse transcriptase that copies their RNA genome into DNA upon infection; and double stranded RNA viruses (dsRNA) (2, 26).

RNA viruses are the most common pathogen infecting humans, animals and plants (10). Over 90% of known plant viruses have an RNA genome (2), and the majority contains ssRNA (26). RNA viruses have been the most successful pathogen since the appearance of cellular life, and this has been achieved by maintaining their genetic variation (24).

1.2 Genetic variability of RNA viruses

The genome of an RNA virus cannot be considered as a single molecular sequence but a dynamic population consisting of thousands of viral mutants, each differing in one or a few nucleotides from the consensus sequence. This intrinsic variability is derived from the error-prone replication process of RNA virus genome (59). The error-rate per replication cycle of RNA viruses is reported to be 0.15 (14), compared to DNA viruses which possess 3'–5' proof-reading and post-replicative repair mechanism have less error rate of 0.003 (15) and are less variable. RNA viruses have great potential for genetic variation due to lack of proof-reading activity of RNA-dependant RNA-polymerase (RdRp); hence reproduction of RNA viruses leads to the generation of individuals that differ genetically from their parental genome. These individuals are referred to as variants or mutants (17). The dynamic distribution of mutants that compose replicating RNA viruses is termed quasi-species (59).

The term quasi-species was introduced by Manfred Eigen and his colleagues to describe the heterogeneity nature of an RNA virus population (59). This concept predicts that a single virus isolate is not a single RNA sequence but a mixture of mutant sequences averaging around a consensus sequence (38). A consensus sequence represents the average sequence at each genome site. An RNA virus genome also consists of a master sequence, which represents the most fit genome sequence under a given environment, and a large number of competing mutants (quasi-species) (59). Domingo (8, 9) defines viral quasi-species as a complex distribution of non-identical, but related viral genomes subjected to a continuous process of genetic variation, competition and selection. A

change in the environment brings about changes in the master sequence and the overall composition of quasi-species changes (24, 59).

Biological selection acts upon quasi-species to allow variant with highest fitness to arise and predominate in the population. When the host or other environmental characteristic changes, such selection pressure is overcome by changing the predominant sequence of the RNA genome. Mutants are selected based on the prevailing condition (24). Generation of quasi-species enables RNA viruses to persist in their host under different conditions (15). This also makes their control difficult, especially in complex mammalian hosts where viruses are always faced with changing conditions such as cell types, immune responses and inflammatory responses; hence they are subjected to different selection pressures which drive high rate of virus genetic variability. A good example is the persistent nature of *Human immunodeficiency virus* (HIV). It has been estimated that 10^{10}- 10^{20} new virions are produced each day in an HIV–1 infected patient (5). This astonishing high rate of new mutants is attributed primarily to polymerase nucleotide misincorporation during replication (8). As infection progresses, continuous production of antigenic variants facilitate escape from host immune responses and antiviral drugs; hence the virus get adapted to any selection pressure. This poses a major obstacle to the design of protective vaccine (18).

Many studies on the genetic variability of plant RNA viruses have reported the heterogeneity nature of the pathogen (3, 14, 15, 20, 29, 41, 54). The increase in the number of papers published on incidence of plant viruses in new geographical areas and the emergence of new plant viruses or plant viral diseases also suggest high heterogeneity of plant RNA viruses. RNA viruses, the largest fraction of all known plant viruses,

display a high level of genetic variation primarily because of high error rate of replication (42). Variants within the quasi-species population of plant RNA viruses are different in their biological properties such as symptoms they cause in different host plant species, their host range, vector transmission properties or geographical locations (15). According to Roossinck (54), plant RNA viruses with broad host range are highly variable while those with narrow host range have little variation. Moreover, Martelli *et al* (41) reported the genetic variation exhibited by members of *Flexiviridae*. Komatsu *et al* (29) also reported the heterogeneity of *Potato virus X* (PVX).

1.3 Mechanisms of genetic variability of plant RNA viruses

Genetic variation in plant RNA viruses primarily results from errors occurring during replication of genomes. Two main mechanisms of genetic variability in plant RNA viruses are:

A. Mutation

B. Genetic exchange

A. Mutation

Mutation, the primary source of genetic variability in plant RNA viruses, is the process by which nucleotides that are not present in the template are incorporated in the daughter strand during the genome replication (38, 51). Mutation rate is the actual rate of nucleotide misincorporation by polymerase error while mutation frequency refers only to those misincorporations that become established in the population (54).

It has been estimated that *Tobacco mosaic virus* (TMV) exhibits 0.10 – 0.13 mutation rate per genome and this rate is applicable to other plant RNA viruses (15). The high mutation rate displayed by TMV and other plant RNA viruses is mostly due to the need for rapid replication of their RNA genome rather than being an evolutionary strategy (15). Mutation rate may be similar for all RNA viruses, but mutation frequency differs for different viruses and this could be reflected in their host range. *Cucumber mosaic virus* (CMV), which has a high degree of mutation frequency, has a very broad host range, whereas potato leafroll virus display low degree of mutation frequency and has a very narrow host range (54). In other words, greater mutation frequency allows the virus to adapt to a higher number of different plant hosts. However, excessive mutation can be detrimental to a virus (9).

Mutation can be point, insertion, deletion or substitution. Higher percentage of mutations in plant RNA viruses are insertions and deletions, and majority involve three to many bases (15). Point mutation refers to a change in a single base of viral genome sequence (51). This is particularly frequent among the RNA viruses and occurs during replication, owing to lack of proofreading activities of RdRps (21).

Insertion mutation involves the insertion of large RNA fragment due to recombination or insertion of few non-template nucleotides into the complementary strand during replication due to errors at template regions that are more difficult for copying by the replicase enzyme (38).

Deletion mutation is a process in which a section of RNA is lost or deleted from the parental genome. Deleted fragment vary from a few nucleotide to a large portions of

the RNA molecules. This does not involve regions that are essential for RNA stability in the host (38).

Substitution mutation involves the exchange of a base for another and this is mainly due to lack of proof reading activities of RdRp. There is one or more base substitution(s) for every replication cycle in RNA viruses (38).

RNA viruses have large and highly diverse populations because of high mutation rate and high accumulation levels in their host; hence they respond easily to selection pressure and survive in changing environment (17). Mutation influences host-virus interaction because it causes changes in viral replication, movement, accumulation and stability of the assembled virions, and vector transmission. This can be linked to the changes observed in the phenotypic characteristics of plant viruses. Mutation plays an important role in plant virus evolution (38, 51).

B. Genetic exchange

This is a process whereby fragment of nucleic acid are switched between different templates and this could be recombination or reassortment.

Recombination

Recombination is a process whereby segments of a genome are switched between the nucleotide strands of different genetic variants during replication (14). Recombination event that occurs between identical viral RNAs is referred to as homologous recombination while non-homologous recombination occurs between RNAs that share no sequence homology (54, 21). Recombination is one of the major factors that results in genetic variation of plant viruses, contributing to the quasi-species structure of RNA

viruses and expanding host range (3, 44). It is an important factor that shape virus genome leading to emergence of novel variants and resistance-breaking strains (3). During mixed infection, which is mostly observed in field plants, related viruses can exchange genes generating more fit variants (54). Furthermore, recombination functions in genome repair (44). It allows two viral genomes with different lethal mutation to regenerate a functional genome or a genome better adapted to its environment, and this is achieved by homologous recombination with error-free parts of co-infecting genomes (54). Non-homologous recombination is responsible for gene rearrangement and insertion of genes from host cell into the viral genome (21).

Positive strand RNA viruses do not recombine as frequently as do DNA viruses, and recombination appears to be rare among the negative strand RNA viruses (24). Recombination rate varies among viral taxa. Chare and Holmes (3) reported significant recombination in plant RNA viruses such as *Rice tungro spherical virus*, member of *Sequiviridae*, *Citrus tristeza virus*, *Closteroviridae* family, *Potato virus X*, member of *Flexiviridae* and most importantly among the members of *Potyviridae*. The analysis of 975 capsid gene sequences and 157 complete genome sequence from different positive strand plant RNA viruses showed that recombination occur at a relatively high frequency (3). In addition, Krause-Sakata *et al* (30) revealed intra-specific recombination between different isolates of *Lettuce mosaic virus* (LMV) and discovered LMV-Tn2, a naturally occurring recombinant between 2 different LMV isolates. Furthermore, based on full genome analysis of 15 *Papaya ringspot virus* (PRSV) isolates, Mangrauthia *et al* (39) demonstrated that recombination plays a significant role in shaping PRSV genome, 12 of the PRSV isolates analyzed showed evidence of recombination. Recombination had been

shown to be essential in the generation of variation observed in the genome organization of *Luteovirus* subgroups (54). Smith *et al* (56) showed that *Sugarcane yellow leaf virus* (ScYLV) arose by recombination between members of the three genera that made up *Luteoviridae* family. Open reading frames (ORFs) 1 and 2 of ScYLV-Florida isolate are most similar to ORFs 1 and 2 of *Polerovirus*, ORFs 3 and 4 are similar to those of *Luteovirus* sequences, and ORF 5 is closely related to that of *Enamovirus*. Recombination plays a direct role in the emergence of some *Tobraviruses* (3), and a recombinant of the type species, *Tobacco rattle virus* (TRV), has been isolated in nature (37). Moreover, phylogenic analysis revealed that recombination has been important in the evolution of *Caulimovirus*, a double-stranded DNA plant virus that replicate by reverse transcription of single-stranded RNA intermediate (54). Padidam *et al* (47) showed that recombination plays an important role in the evolution of geminiviruses, single-stranded DNA viruses, and occurs between and within species and across genera.

Recombination can create new viruses especially by acquisition of a genetic element from cellular nucleic acids or from other viruses (24). *Hepatitis D virus* (HDV), an animal virus, contains an ORF encoding delta antigen protein. Liver cells have been found to express a cellular homolog of the delta antigen, suggesting that HDV may have arisen by the acquisition of a cellular RNA transcript by a viroid-like RNA (24). Moreover, a recombinant of *Turnip mosaic virus* (TuMV) a member of the genus *Potyvirus* was found to contain sequences of a host gene, its 3' -467 nucleotides sequence was highly homologous to a part of the chloroplast ribosomal protein 12 gene (37). Recombination occurred between wild-type *Cauliflower mosaic virus* (CaMV) and viral transgene derived from gene VI of CaMV D4 strain. These recombinants were found to

cause different symptoms and became systemic in host that the wild-type virus could only locally infect (37, 64). Thus recombination is important in transgenic resistance and caution must be taken in the use of viral sequences to provide transgene-mediated resistance. As the acquisition of cellular genes shape the virus evolution so does the acquisition of virus gene shape the host evolution (24).

Besides generation of more fit variants, recombination may also results in changes in biological properties of the virus with major epidemiological consequences such as, appearance of resistance-breaking strains, enhanced pathogenicity and acquisition of broader host range (15, 44).

Reassortment

Genetic exchange between two viruses also results from reassortment, which involves the exchange of segments between viruses with split genome which coexist in the same host. The resultants are called reassortants (14, 21). The genomes of many RNA viruses consist of segmented parts and this is a strategy for gene organization and expression (54). Reassortment provides a mechanism for variation and generation of new viruses especially in host plant with mixed infections that are common in nature. Evidence for reassortment in natural populations of plant viruses has been reported (14, 54). Isolation and characterization of naturally occurring reassortants among the members of *Cucumovirus* genus had been reported. One reassortant contained RNAs 1 and 2 from *Peanut stunt virus* (PSV) and RNA3 from CMV which also contains 5' and 3' ends of PSV (62). Reassortants containing genome parts of CMV and *Tomato aspermy virus* has been reported to occur in nature (37). Garcia-Arena *et al* (14) also reported natural

reassortment among CMV strains. Two reassortants between members of *Tobravirus*, TRV and *Pea early browning virus* (PEBV), has been described. The RNA2 segment of these reassortants contained the coding sequence of PEBV, but the 5' and 3' ends from TRV (52).

Nevertheless, reassortment is not a common event among plant RNA viruses. However, despite rare occurrence, its impact on the evolution of new viral species and expansion of host range is remarkable. Reassortment also plays an important role in evolution of animal viruses, for example, periodic antigenic shift of *Influenza A virus* resulting from gene segment reassortment causes emergence of new influenza A virus and initiate new human pandemics (24).

RNA viruses undergo genetic rearrangements that allow exchange of corresponding genes or gene segments during mixed infection. These recombination and reassortment events allow the most efficient and environmentally adapted combinations of genes to emerge from the available genetic pool, increasing the potential for viral survival (24).

1.4 Selection

The processes that determine the distribution of genetic variants generated by mutation or genetic exchange are selection and genetic drift. Selection is the process by which the frequency of the fittest variant in a given environment increases within the population (positive selection) while the frequency of the less fit variant decreases within the population (negative selection) (14). Viral fitness has been defined as the relative ability of a virus population to produce infectious progeny under defined environmental

conditions (7). Fitness measures the degree of adaptation and ability to replicate in a defined environment. Fitness may vary in different individuals of the same host species. Biological environment vary with time within each infected individual; plant viruses that infect wide range of hosts, or that are transmitted by different vector species face more diverge environment and hence different selection constraints. In addition, plant viruses that replicate in both plants and in their insect vectors have dramatically different selection pressure in each host (54).

Selection is associated with every factor in virus life cycle. Selection pressure is associated with the maintenance of intrinsic properties of virus, such as functional structures that are important for virus replication. In tobamoviruses, selection pressure is for maintaining the coat protein (CP) (15).

Selection is also associated with the host plants; here a virulent strain that decreases the fitness of a host is selected for or against within the population (14). Since viruses depend upon their hosts for replication and survival, any viral variant that damages the host significantly would be selected against. Nevertheless, a variant that changes the host range would be advantageous (26). Documented evidence of host-associated selection includes consistent selection of different variants in different host plants (14). Moreover, host-associated selection is related to overcoming resistance genes. Selection for virulence determinants in *Beet curlyt topvirus* (BCTV) and *Rice grassy stunt virus* have been reported (15).

Other selection factors are those associated with the interaction of viruses and their vectors. It has been proposed that geographically related antigenic variation in

begomoviruses is an evidence of vector selection (22). In addition, selection of variants from a mixture of cucumoviruses by aphid may also be evidence of vector selection (16).

1.5 Implications of genetic variability of RNA viruses

An RNA virus population consists of dynamic mutants' spectra, a quasi-species population, rather than a defined genomic sequence (6). Many variant genomes are generated in any natural infection by an RNA virus; this allows maximal variability, adaptability and successful colonization of their hosts. RNA viruses take the advantage of their inbuilt variability to adapt through selection to environmental changes. RNA viruses explore their error copies to escape host's defense mechanism and antiviral drugs. In animal virus for example, HIV mutant with high level of resistance was isolated from a patient receiving antiviral therapy and was shown to involve amino acid changes in residues located at the inhibition binding site (6). Likewise, high variability observed in *Human influenza A virus* is driven by immune-selection. The virus evolves rapidly that new variants can infect a population that had become immune (18).

Furthermore, pathogen-derived resistance, a major strategy for controlling plant virus diseases, in one case did not last long when tested in the field due to generation of variants that overcame such resistance (16). Recombinants between transgene RNA and RNA virus challenging the host has been reported (16). Through quasi-species, RNA viruses refine their genetic structure and adapt to changing environment especially when moved from one host to the next. This makes their control difficult; an attempt to control RNA viruses provides new selection pressure (54). A greater understanding of the genetic

variability of RNA viruses and their evolution will provide a better and longer-lasting control of virus diseases.

As RNA viruses continuously undergo variation, a point is reached where variants become so phenotypically and genotypically different that the variant becomes separated as a new entity (59). Genetic variability leads to emergence of new viruses. New human, animal and plant viruses emerge through mutation, recombination and reassortment (53). A good example is the *Influenza virus* which rapidly generates new strains with significantly altered virulence via recombination and reassortment (33). Li *et al* (33) speculated that bird flu strain of influenza H5NI will mutate and emerge as a widespread virus among human population. Furthermore, viruses due to their heterogeneity have jumped from a reservoir host to a new host with more serious disease effect (53). Such emergent human viruses include HIV, *Coronavirus* associated with severe acute respiratory syndrome (SARS) and, *Ebolavirus* and *Marburgvirus* are thought to have emerged from viruses in animals (13, 58). It has been speculated that future viral diseases will be caused by RNA viruses due to the rapid evolution potential of their quasi-species (24). Tospoviruses and tobraviruses had been reported as emerging plant viruses. Tospoviruses evolved from animal *Bunyvirus* and within this family, they are the only group infecting plants. The wide host range of their vector, western flower thrips, has also contributed to the evolution of tospoviruses (24, 54). In addition, emergent plant viruses such as members of *Begomovirus*, members of *Crinivirus* and *Pepino mosaic virus*, a member of *Potexvirus* genus have been reported (53). Plant RNA viruses will continue to emerge as long as there is a continuous change in the environment and

climate, expansion of hosts and insect vectors, changes in agricultural practice and movement of human population and plant products (24, 54).

Genetic variability of RNA viruses may influence viral diagnosis; mutation at the antibody binding site may influence the outcome and may lead to a false negative or positive results. Mutation in the nucleotide sequences can prevent binding of the PCR primers to target sequences and hence, may result in binding to non-target sequences (61) or failure of amplification (57). Insertion or duplication of genes or sequences can cause size variation in PCR product. Variation can also cause strains of the same virus to have significantly different biological properties such as symptoms, particles size and pathogenicity (61).

1.6 Molecular methods for studying genetic variability of RNA viruses

There are various ways by which genetic variability can be studied, these include the following:

A. Polymerase Chain Reaction (PCR)

PCR is an automated process that exponentially amplifies DNA, and over many cycles generates millions of copies of specific DNA nucleotides. It starts by denaturing double-stranded DNA to form single strands, each containing full length of target DNA. Upon cooling both the forward and reverse primers anneal to their complementary sequences on different strands. Polymerase synthesizes new strands by adding nucleotides to the 3' end of each primer. The synthesis of new DNA is in the 5' to 3' direction. For each cycle of heat denaturation, annealing and synthesis, the region between the primers is copied.

PCR has numerous applications. It is most commonly used in molecular analysis such as for amplification, mutagenesis and sequencing of genes. PCR is an important tool in the study of genetic variability within a particular viral species, genus or family. Gel electrophoresis of PCR products reveal variation between isolates based on molecular weight of the amplified DNA fragments.

Sequencing of PCR product is used to determine variability among viral isolates. This reveals up to a single difference in the genome of virus isolates under study. Moreover, sequence analyses not only reveal variation among isolates, but also provide information on molecular processes responsible for sequence variation and evolutionary phenomena. Comparison of sequences reveals variation among isolates; and the percentage identity or differences can be estimated. Of the molecular approaches, DNA sequencing is the most widely used method for inferring phylogenetic history of viral isolates. The study of DNA sequences account for 50% of all molecular investigations of systematic (23). Intraspecific sequence variation can be used to study epidemiology of a disease (25), gene evolution and geographic variation (23).

B. Restriction fragment length polymorphism (RFLP)

Genetic variability resulting from base substitution, insertion and deletion events can be detected by digestion with restriction endonucleases. Mutation can create or eliminate recognition site of a particular endonuclease, which may change the numbers and sizes of fragments created by such enzymes. A restriction endonuclease cleaves a DNA fragment at a characteristic recognition site, typically 4-6 base pairs long or more. Restriction enzyme specificity is revealed by complete digestion of a particular DNA

molecule following cleavage at recognition site, and consistent yield of an array of fragments. Such fragments are sorted out according to their size by gel electrophoresis. The variation in fragments sizes and numbers as sorted by gel electrophoresis following digestion with restriction enzyme are referred to as RFLP (23).

In addition, besides the ability to reveal genetic variability, RFLP also severs as an important method for the identification and classification of viral isolates. RT-PCR followed by RFLP has been used to identify and classify *Alfalfa mosaic virus* (AMV) isolates into two subgroups (49). Marie-Jeanne et al (40) utilized RT-PCR and restriction digest to differentiate potyviruses infecting *Poaceae*. Xu and Nie (66) used RFLP as a confirmatory test for PCR analysis of AMV in potato.

C. Phylogenetic analysis

Phylogenetics is the study of the evolutionary history of organisms, including viruses, using tree-like diagrams to represent pedigrees (18). Phylogenetic analyzes differences at various positions in viral sequences and infers hypotheses about the evolutionary relatedness of the nucleotide or amino acid sequences. Based on the sequence similarity of nucleotides or amino acids, evolutionary relationship between viruses can be inferred.

Phylogenetic analysis is an important tool in determining the factors that influence worldwide variation in a single virus species. Phylogenetics has been used to understand the variability and the seasonal epidemic of *Human influenza A virus*. Phylogenetic analyses of 900 complete genome of *Human influenza A virus* from northern and southern hemisphere revealed that the frequent migration of the virus across

the hemispheres contribute significantly to the genetic variability and the introduction of new influenza epidemic in both hemispheres (45). In plant RNA viruses, phylogenetic surveys have revealed recombination as an important factor that facilitates variability and contributes to the evolution of plant RNA viruses, and more significantly, among the potyvirus (3).

Phylogenetic analyses help to reveal the source, natural reservoir or ancestor of viruses. This is important in developing ways to combat viruses. The source of HIV-1 was revealed by phylogenetic analysis to be mangabey (old world monkey) which is commonly kept as household pet and serve as a source for food in the West Africa. Mangabey scratches, bites or blood contact by other means had been thought to be responsible for transmission of the virus to humans (18). Phylogenetic analyses of TuMV, a RNA plant virus that occurs worldwide, showed correlation between the genetic variability and geographical origins, and revealed that majority of the genetic diversity within viral population is a results of recombination (46). Phylogenetic analyses of plant viruses with TGB revealed co-evolution of species within the genera and convergent evolution of species among the family (65).

Phylogenetic analysis is important in determining the taxonomy position of viruses at genera and family levels. The establishment of *Flexiviridae* family was strongly based on the phylogenetic relationship among polymerase and CP genes of member species (1). Information revealed by phylogenetic analyses, such as relatedness, evolutionary pattern, factors that influence the genetic diversity, source and natural reservoir, is important for the design of strategy for controlling plant viruses.

1.7 *Flexiviridae*

Flexuous plant viruses, *Flexiviridae*, *Potyviridae* and *Closteroviridae*, constitute the largest fraction of known plant viruses causing diseases in crops. Members of these three families are single-stranded, positive-sense RNA viruses (27). Members of the family *Flexiviridae*, and in particular the large genus *Potexvirus*, are significant to agricultural productivity (1, 27).

The family *Flexiviridae* was recently recognized by international committee on taxonomy of viruses (ICTV), and its establishment was based on common feature of genome organization and phylogenetic relationship among replicase and CP genes (41). Member species of the *Flexiviridae* family possess monopartite, 3'-polyadenylated genome. The genome contains basically 3 to 5 genes which encode at least 3 functional proteins. The replicase, product of the first and largest gene, function in the genome replication. CP is the product of the penultimate gene, which encapsidates an RNA molecule of 6–9 kb in size (1, 41). In addition, CP sequence analyses serve as the molecular criteria for demarcation of species within the family (1). The CP of flexiviruses share the conserved structural core and evolutionary origin with the CP of *Potyviridae* and *Closteroviridae* (41). *Flexiviridae* possesses two unrelated types of movement proteins (MP) encoded by the central gene(s), and are involved in viral cell-to-cell movement. Viruses with three MPs posses TGB proteins while those with one MP possess a 30K-like protein (41). Sequence analysis of TGB and 30K MP showed they are quite distinctive and suggest a convergent evolution; however, sequence analyses of replicase and CP suggest that the viruses within *Flexiviridae* family form a coherent

taxonomic unit (1, 34). Table 1.1 shows genera and type species within the *Flexiviridae* family.

Member species of the *Flexiviridae* family are transmitted by mechanical means, activities of vectors and by seed transmission. Members of *Potexvirus* genus do not require insect vectors and are transmitted mainly by mechanical means (41). Hence, are subjected to less selection pressure and are less variable compared to those with vector-associated selection pressure. However, PVX, the type species of *Potexvirus* has a wide host range (60) and strains induce diverse responses in host plants (29). Komatsu *et al* (29) reported that variation in pathogenicity and symptoms of each strains of PVX are determined by small number of nucleotides. Species within the *Potexvirus* genus are demarcated based on the percentage identity of the CP core region sequences. Distinct species has less than 65% sequence homology with unrelated species (1).

1.8 *Hosta virus X* in hosta

In the USA, several viruses have been reported to infect hosta such as *Arabis mosaic virus*, *Tomato ring spot virus*, *Impatiens necrotic spot virus and Hosta virus X* (HVX) (Table 1.2). HVX, the most widely spread virus in hosta, has a high economic impact (35). HVX, a positive single-stranded RNA of 6.4Kb in size, is a member of the genus *Potexvirus* (48). Its flexuous rod-shaped particles, belongs to *Flexiviridae* family. The virus particles are of 540nm in length and 13nm wide. The viral genome possesses five ORFs (Figure 1.1). The 5'–proximal ORF1 encodes a 165 KDa protein, RNA-dependent RNA polymerase, involved in the replication of the viral genome. The central ORFs 2, 3 and 4 overlap, known as TGB encode 26, 13 and 8KDa proteins, respectively;

they are involved in the cell-to-cell movement of the virus in infected plant. The 3'–proximal ORF5 encodes the 23KDa viral CP required for virion assembly as well as virus cell-to-cell movement (48, 60). Based on CP sequence percentage identity a Korean isolate of HVX (HVX Kr) is closely related to *Papaya mosaic virus*, *Alternanthera mosaic virus and Cactus virus X* (48).

HVX has a narrow host range; it naturally infects only hosta and experimentally infects *Nicotiana benthamiana* (4). HVX is commonly transmitted through any mechanical means that involve the transfer of sap from infected plant to a susceptible plant, especially when contaminated propagation equipments are used on susceptible healthy hostas (36). It can also be transmitted through seed from infected parental plants to the offsprings (55). There is no report or evidence that HVX is spread by an insect vector. Hosta cultivars respond differently to HVX infection and symptoms range from severe to symptomless (36). In some cases, it takes a year or more for symptoms to develop (28, 63). Symptoms with HVX infection include mottling, puckering, necrosis, crinkling twisting and stunting.

HVX was observed in small nurseries in the 1900s (63). Propagation of HVX infected hosta led to the rapid spread of the virus. Before the virus was widely known, HVX infected hostas with symptoms such as mottling and spotting were thought to be the result of color mutation and were assumed to be new hosta varieties. These strange cultivars were propagated, named and sold as new highly prized varieties. This virus spread from nurseries to wholesale hosta supplies in Holland and USA, and are thought to be the main source of HVX epidemic (28, 63). Wholesale growers and the hosta industry in Holland lost thousands of hostas due to eradication program targeting the

infected hostas (63). HVX was first isolated and characterized in 1996 by Dr Lockhart, University of Minnesota (35).

HVX is considered to be the most important pathogen of hostas and has been a concern to nurseries, garden centers and home gardeners. Lack of proper control of HVX could cause the virus to spread unnoticed in nurseries and gardens resulting in great economic loss. Most garden centers and nurseries employees do not recognized the symptoms of HVX infected hosta. Similar to any other plant disease caused by a virus, there is no cure for HVX infected plants. The virus is symptomless in some cultivars and difficult to detect in many others. This could be due to the genetic variability of HVX. As such, this virus presents challenges for hosta growers.

1.9 Economic significance of hosta

Hosta is an herbaceous perennial plant of the family *Hostaceae* and is the most popular selling perennial plant in USA (19). Hosta is a native of Korea, Japan and China, and was thought to have evolved from lily-like ancestors (19). Hosta was brought to the USA in the middle 1800s. The production value of hosta in the USA is $25million per year while the annual production value in Tennessee is $1.5million (Mark Windham, Personal communication). Hosta grows under a wide range of climatic conditions, and tolerates both sunny and shady environments. They are used as ornamental and landscape plants due to their attractive foliage, diversity of shapes, sizes and colors. Hosta has replaced roses as the most popular landscape plant in the USA (36). Hosta is easily grown and maintained, and is widely available in nurseries and garden centers. There are over 2,500 different cultivars currently available. Hosta is propagated vegetatively and by seed

(19). Virus-like disorders have been a threat to hostas and render them undesirable as landscape plants.

1.10 Research objectives

Studying genetic variability of HVX is the main focus of this research with the goal of establishing a sensitive diagnostic system for HVX in hostas. Understanding the genetic variability of virus is important for accurate diagnosis, epidemiological investigations, screening for resistant cultivars and design of control strategies. There is no information on the genetic variability of HVX, but it has been shown that the sequence variability of 5' and 3' untranslated regions (UTR), and CP of PVX affect the pathogenicity of the virus (11).

This research had three main objectives: 1) To determine the presence and incidence of HVX in the State of Tennessee; 2) To determine the genetic variability and phylogenetic relationship of HVX isolates; 3) To develop an sensitive assay for HVX in large scale screening.

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Appendix

Appendix 1: Tables

Table 1.1 Genera and type species within *Flexiviridae* family (1, 41)

Genus	Type speices
Potexvirus	*Potato virus X*
Mandarivirus	*Indian citrus ringspot virus*
Allexivirus	*Shallot virus X*
Carlavirus	*Carnation latent virus*
Foveavirus	*Apple stem pitting virus*
Capillovirus	*Apple stem grooving virus*
Vitivirus	*Grapevine virus A*
Trichovirus	*Apple chlorotic leafspot virus*

Table 1.2 Viruses that naturally infect hostas (32, 35, 55)

Virus	Genus/Family
Impatiens necrotic spot virus	*Tospovirus* / *Bunyaviridae*
Tomato spotted wilt virus	*Tospovirus* / *Bunyaviridae*
Tobacco ring spot virus	*Nepovirus* / *Comoviridae*
Arabis mosaic virus	*Nepovirus* / *Comoviridae*
Tomato ring spot virus	*Nepovirus* / *Comoviridae*
Tobacco rattle virus	*Tobravirus* / *Unassigned to family*
Hosta virus X	*Potexvirus* / *Flexiviridae*

Appendix 1: Figure

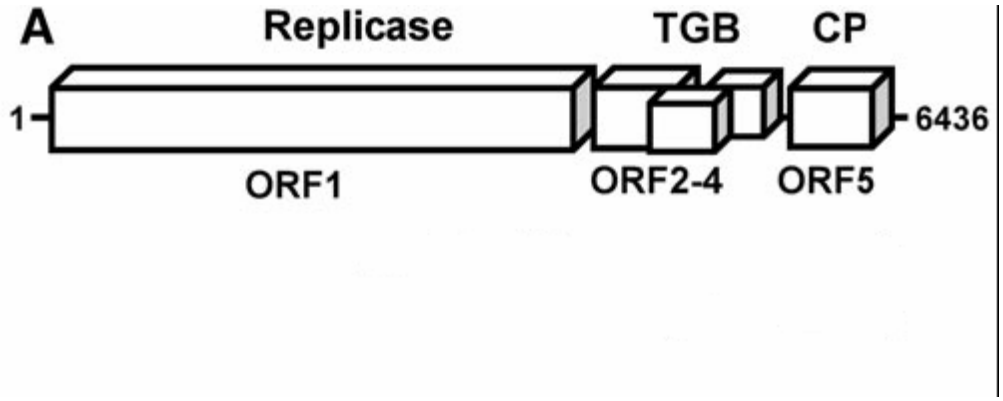

Figure 1.1 *Potexvirus* genome organization (31)

Symbols indicate Triple gene block (TGB) and coat protein (CP). ORF indicates Open reading frame.

Chapter II

Incidence of *Hosta virus X* in Tennessee, Genetic Variability and Phylogenetic Analysis

Adedire, O. L. Wen, R.H., Windham, A., Windham, M. and Hajimorad, M.R. 2009. Hosta virus X in Hosta identified in Tennessee, USA. Plant Pathology 58:405

Abstract

In the United State of America (USA), several viruses have been reported to infect hosta. *Hosta virus X* (HVX), a member of the genus *Potexvirus* belonging to the family *Flexiviridae*, is the most prevalent and economically important virus. The virus limits the production of hosta worldwide. In this study, HVX isolates from naturally infected hosta cultivars were obtained from different geographical regions of Tennessee. Eighty-two hosta plants exhibiting various virus-like symptoms including cultivars 'Sun and Substance' 'Lady Guinevere', 'Golden Standard', 'Frances Williams', 'Antioch' and 'Pilgrim' were sampled and screened by squash immunoblotting assay. Sixty-one plants representing twenty-five cultivars were found to be infected with HVX. Biological assays and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) were carried out on selected HVX-infected hostas. The widespread presence of HVX was established in the State of Tennessee based on the high rate of infection. To detect possible genetic variability among the isolates, triple gene block (TGB) 1 and coat protein (CP) gene sequences of thirty isolates were determined. The sequence data were compared with available published TGB1 and CP sequences of HVX present in GenBank. Multiple alignments of CP gene sequences revealed 98.3–100% and 98.6–100% nucleotide and amino acid sequence identity among HVX isolates, respectively. TGB1 sequences of HVX isolates shared 97.4–100% nucleotide sequence identity, and 97–100% amino acid sequence identity. TGB1 of the Tennessee isolates were found to be three nucleotides and one amino acid longer than that of HVX Korean isolate (HVX-Kr). Phylogenetic analysis of HVX isolates and representative species of *Flexiviridae* was carried out using TGB1 and CP sequences separately or combined. Analyses confirmed the taxonomic position of

HVX in *Flexiviridae*, and showed that HVX is closely related to *Plantago asiatica mosaic virus*, *Tulip virus X* and *Cassava common mosaic virus*. Phylogenetic analysis revealed low genetic variability among HVX isolates irrespective of geographical location and host cultivars from which they were isolated. Analyses showed close relation among Tennessee, Korean and US isolates, probably indicating recent derivation from a common ancestor. However, substitutions observed among isolates of HVX are not shared, likely attributed to HVX being a newly derived virus

2.1. Introduction

RNA viruses have high potential for genetic variation. This enables them to adapt to changing environments (10). Genetic variability of plant RNA viruses is being studied to establish their evolutionary history and most importantly to design strategies for effective diagnosis and control of diseases they cause (39, 21). Moreover, the durability of plant resistance to viruses is determined by the variability potential of the virus population (10). Complete genomic sequences of many species of plant viruses have been determined and their genetic variability and phylogenetic relation have been inferred (19, 4, 12).

*Hosta virus X (*HVX), a positive-sense ssRNA of 6.4Kb in size, is a member of the genus *Potexvirus* within the family *Flexiviridae*. The genome of HVX, like any other potexvirus possesses five open reading frames (ORF). The 5'-end of the genome has a methylguanosine cap and the 3'-end has a poly (A) tail (34). The first and largest ORF is the polymerase gene, which is involved in the replication of the genome. Downstream of the polymerase, are three overlapping ORFs known as TGB 1, 2 and 3. The TGBs encode

proteins that function in concert to mediate virus cell-to-cell movement (22). Besides the movement function, TGB1 of *Potato virus X* (PVX) possesses RNA silencing suppressor activity (2). Moreover, TGB1 of potexviruses has RNA helicase activity, promotes translation of viral RNA and increases the permeability of plasmodesmata (33). The TGB2 and TGB3 proteins are endoplasmic reticulum–associated proteins and are also required for virus movement (34). Potexviruses require TGB proteins and CP to facilitate viral cell-to-cell movement and vascular transport (33, 14). Movement proteins are essential for the successful establishment of a virus in a host plant and development of systemic infections (13). The efficiency of movement proteins could be related to the virus pathogenicity, virulence and host range (36). The 3'-proximal ORF 5 encodes the viral CP, which is required for the genome encapsidation and it is also involved in the viral cell-to-cell movement (33, 18)

HVX is the major limiting factor for hosta production worldwide (15). HVX naturally infects only hosta and it is transmitted by seed and mechanical means. Symptoms induced by HVX include mosaic, necrosis, leaf distortion as well as leaf deformation and reduced plant growth (16, 26). Although reports on the presence of HVX in hosta from elsewhere have been published, the incidence of HVX in Tennessee (TN) and the genetic variability of the virus in general have not been reported. In this study, the prevalence of HVX in TN was established. In order to determine the sequence variability among HVX isolates, nucleotide and amino acid sequences of CP and TGB1 of HVX isolates collected from different geographical regions of TN were analyzed. Sequences of the previously reported HVX isolates and representative species from all genera within the *Flexiviridae* family were obtained from the GenBank. These were

compared with HVX sequences obtained from TN isolates and the phylogenetic relationship was determined.

2.2. Materials and methods

A. Collection of hostas with HVX-like symptoms from regions in TN

Hostas of different cultivars that were naturally infected with HVX were used for this study. During fall 2007 to fall 2008, a total of 31 different hosta cultivars with HVX–like symptoms were collected from the three geographical regions of TN. Infected hostas were collected from Knoxville, Johnson City, Morristown and Greenville representing East Tennessee. Infected hostas from the Middle Tennessee were obtained from Franklin, Smyrna, Nashville and Murfreesboro; whereas those from the West Tennessee were obtain from Jackson and Memphis (Table 2.1). A total of 82 infected plants (Table 2.2) were obtained from different nurseries, local growers, gardens and retail outlets. Symptomatic hostas were also obtained from the UT Soil, Plant and Pest Center in Nashville. Figure 2.1 shows different symptoms of HVX in naturally infected hosta plants.

Infected hostas were maintained in a light–temperature–controlled growth chamber (Environmental Growth Chamber, USA) at 25^0C with 16 hours light: 8 hours dark photoperiod cycle and 46% humidity. Asymptomatic hostas obtained were tested for the absence of HVX using Agdia HVX ImmunoStrip Kit at the point of collection as instructed by the manufacturer. These hostas were maintained separately and used as negative controls in subsequent experiments.

B. Diagnosis of HVX

1. Serological assays

All the collected hosta cultivars were indexed for the presence of HVX by serological tests. Antiserum against HVX CP, kindly provided by Dr. B.E.L. Lockhart (University of Minnesota, USA), was used in all the serological tests conducted in this thesis. Serological tests were carried out using squash immunoblotting as well as western immunoblotting assays.

Squash immunoblotting assay

All plants obtained were screened for the presence of HVX by squash immunoblotting assay according to Van Regenmortel and Dubs (32) protocol with minor changes. Antibodies against HVX CP, and alkaline phosphatase conjugated goat anti–rabbit IgG (Sigma-Aldrich Missouri, USA) were diluted in Tween-20 Tris Buffer Saline (TTBS) at pH 7.5 (0.05 % Tween-20 in Tris Buffer Saline (TBS): 20 mM Tris, 500mM NaCl) + 5% non–fat milk. Leaf tissues from each hosta were squashed to release sap on nitrocellulose (NC) membrane (Schleicher Schuell Dassel, Germany). The membrane was incubated for 90 min in a blocking solution (5% non–fat milk in TBS pH 7.5). After washing the membrane twice in TBS, it was incubated for 90 min in diluted HVX antibodies at the ratio of 1:1000 (v/v). After washing the membrane again, it was incubated for 60 min in diluted alkaline phosphatase conjugated goat anti–rabbit IgG (Sigma–Aldrich) at the ratio of 0.6:1000 (v/v).

NC membrane was washed four times for 5 min each with gentle agitation in TTBS after incubation in antibodies. Membrane incubation was done at room temperature in small polyethylene bag sealed and placed on a belly dancer shaker (Stovall Life Science Inc; USA) for gentle agitation. The membrane was developed with 10 ml of substrate buffer (100mM Tris, 100mM NaCl and 5mM $MgCl_{2;}$ pH 9.5) containing 33µl of nitroblue tetrazolium (Promega California, USA) and 16.5 µl of 5–bromo–4–chloro–3 indolyphosphate (Promega). The development reaction was observed for any changes in color. Finally the blot was immersed in distilled water to stop the development reaction. It was air dried at room temperature and store in 1ow light density (Figure 2.2).

Western immunoblotting assay

Western immunoblotting was carried out on a representative HVX isolate where it served only as a confirmatory test.

Discontinuous Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS – PAGE)

SDS-PAGE was used to estimate the molecular mass of HVX CP according to Sambrook and Russell (28) with minor changes. Symptomatic leaf tissues collected from representative hosta was pulverized in liquid nitrogen and mixed with 5 volume of extraction buffer (10 % glycerol, 50mM Tris-HCl, pH 6.8, 5% 2–mercaptoethanol and 2% SDS). One volume of homogenized sample was mixed with 1 volume of SDS–PAGE loading buffer (62.5mM Tris–HCl pH 6.8 containing 5% 2–mercaptoethanol, 0.01% of bromopherol blue and 10% glycerol) and boiled for 5 min. This was then centrifuged at

10,000 rpm for 10 min. Purified *Alfalfa mosaic virus* (AMV) obtained from Plant Virology Laboratory-The University of Tennessee was included as a control; 2µg of AMV purified preparation was mixed with SDS–PAGE loading buffer 1:1 (v/v) and boiled for 5 mins. Slab minigels (Bio-Rad California, USA) were used for the electrophoresis. Glass plates were assembled, 12% lower resolving gel was poured into the space between the glass plates and allowed to polymerize at 37^{o}C for 1hr. The 5% upper stacking gel was poured on the lower polymerized gel and maintained at 37^{o}C for 30 mins to allow for polymerization of the upper gel. Assembled cassette was submerged into an electrophoresis chamber and was subsequently filled with tris–glycine electrophoresis buffer pH 8.3. Samples (20µl each) were loaded into each of the wells and electrophoresis was done initially at 120V and subsequently at 180V. The electrophoresis was continued until the tracking dye (bromophenol blue) reached the bottom of the gel. The gel was stained in 0.1% coomassie brilliant blue containing 40% methanol and 10% acetic acid. The gel was then destained in 40% methanol and 10% acetic acid, and photographed using White Light Transilluminator Universal Hood from Bio–Rad Laboratory.

Western immunoblotting

Western immunoblotting was conducted according to the method described by Sambrook and Russell (28). Proteins separated by SDS-PAGE were transferred electrophoretically to NC membrane (Schleicher Schuell) using a trans–blot electrophoretic transfer cell (Bio–Rad) in the presence of transfer buffer (20% methanol, 0.1 % SDS, 25mM Tris, 192mM glycine, pH 8.3). The membrane was incubated for 2 hrs

in blocking solution TBS + 5% non-fat milk. The membrane was washed at least 3 times in TBS and incubated for 1 hr in 1:1000 (v/v) dilution of HVX polyclonal antibodies in TTBS+5 % non–fat milk. After washing the membrane four times in TTBS, it was incubated for 1 hr in a solution of alkaline phosphatase conjugated goat anti–rabbit IgG antibodies (Sigma-Aldrich) diluted in TTBS+5% non–fat milk (0.6:1000 v/v). All incubations were carried out at room temperature in a small polyethylene bag placed on a belly dancer shaker (Stovall). The membrane was washed and developed in substrate buffer as described above.

2. Biological assay

Nicotiana benthamiana was used as an indicator plant to assay for presence of HVX in the collected hostas. A nursery of *N. benthamiana* was made with virus-free seeds obtained form the seed collection of the Plant Virology Laboratory–The University of Tennessee. The plants were transplanted and grown in a light–temperature–controlled growth chamber (Environmental Growth Chambers, USA) at 25^0C with a 16 hours light:8 hours dark photoperiod and 46% humidity.

Young symptomatic leaves from HVX–infected hostas were macerated with sterilized cold mortars and pestles in cold extraction buffer (1% K_2HPO_4 and 0.5% Na_2SO_3) at 1:10 (w/v) dilution ratio. The extract was used to mechanically inoculate leaves of *N. benthamiana* which were pre-dusted with carborundum powder (600 mesh) (6). The inoculation was done in triplicates. The inoculated plants were maintained in the growth chamber and observed for the development of symptoms. HVX infection was confirmed by squash immunoblotting assay two weeks post-inoculation.

C. Maintenance of HVX isolates in storage

HVX infected hosta tissues from different regions of TN were stored under two different conditions. Symptomatic leaves from HVX-infected hostas were initially immersed in liquid Nitrogen and subsequently stored at -80^{o}C. Leaf tissues from the same plants were also shredded and placed in Petri-dishes over calcium chloride at 4^{0}C (20).

D. HVX genomic regions used for variability study

Potato virus X (PVX), a type member of *Potexvirus* genus, was used as a model to determine the regions of HVX genome to be utilized for variability studies. There is only one complete genome sequences of HVX currently available in the GenBank from Korea (HVX-Kr), accession number NC_011544. Hence, PVX served as a model system to identify the variable genomic region(s) of the virus. The sequences of five well studied and completely sequenced PVX isolates were obtained from the GenBank. Different regions of the PVX isolates were compared in NCBI database using Blast program and their percentage identities were obtained. PVX isolates used for the comparison, their accession numbers and percentage identities are shown in Table 2.3.

E. Extraction of total RNAs from hosta leaf tissues

Total RNA was extracted from symptomatic leaves of HVX–positive hostas using RNeasy Plant Mini Kit (Qiagen California, USA) following the manufacturer's instructions. Total RNA was also extracted from leaf tissues of HVX-free hostas where it served as a negative control in subsequent experiments. All containers and buffer used

were ribonuclease-free. RNA extracts were quantified using a smart Spectrophotometer (Bio-Rad). The extracts were used immediately or stored at -20^{o}C until needed.

F. Reverse transcription (RT) reaction

RT was carried out according to the method described by Sambrook and Russell (28). Total RNA was used as a template for first–strand complementary DNA (cDNA) synthesis using SuperScript III Reverse Transcriptase (SS III RT) (Invitrogen California, USA). A mixture of 1µl of 10pmol primer (Table 2.4), 10µl total RNA extract, 1µl 2.5mM dNTPs (Takara Shiga, Japan) and 2.5µl distilled water was heated at 65^{o}C for 5 min. After incubation on ice for 2 min, other components were added to the reaction, including 4µl of 5X first strand buffer (Invitrogen), 1µl of 0.1M dithiothreitol (DTT) (Invitrogen) and 0.5µl of SS III RT. This 20µl reaction was incubated at 50^{o}C for 45 min and then heated to 70^{o}C for 15 min in a peltier thermal cycler (MJ Research, Inc; Massachusetts, USA). Three types of primers synthesized by Integrated DNA Technologies, USA were used (Table 2.4). Gene specific reverse primer complementary to the 3' end of HVX genome, oligo (dT) primer complementary to poly (A) and random nanomer primer complementary to all possible sequences. cDNA was also generated using total RNA extracted from HVX-free hosta using oligo dT primer where it severed as a negative control in subsequent experiments. The cDNAs obtained were either used immediately or stored at -20^{o}C until needed.

G. Amplification of CP and TGB1 genes by polymerase chain reaction

Polymerase chain reaction (PCR) was done according to Sambrook and Russell (28) method with minor changes. First–strand cDNAs served as templates for PCR amplification of the targeted genes. Vector NTI program (Invitrogen) was used to design primers for the amplification and sequencing of the complete HVX CP or TGB1 genes based on the HVX genome sequences obtained from the GenBank (Tables 2.4). Amplification reactions were performed with Ex *Taq* polymerase (Takara) using 10X buffer provided. Reaction mixtures of 50µl consisted of 2µl of cDNA template, 2µl of 10pmol forward primer (HVXs5639; Table 2.4), 2µl of 10pmol reverse primer (HVXa6488; Table 2.4), 2µl of 2.5mM dNTP mixture (Takara), 5µl of 10X reaction buffer, 0.25µl of Ex *Taq* and 36.75µl of distilled water. CP fragments were amplified in a peltier thermal cycler (MJ Research) with 1 cycle at 94^{o}C for 4 min, followed by 35 cycles at 95^{o}C denaturature temperature for 30 sec, 56^{o}C annealing for 30 sec and 72^{o}C extension for 30 sec. This was followed by a cycle of 10 min extension at 72^{o}C. Fragments containing TGB1 was amplified in the same manner, except that annealing was done at 62^{o}C and primers HVXs4501 and HVXa5377 were used (Table 2.4). Two negative controls were included in all the PCR reactions: a cDNA generated from HVX-free hostas leaf tissues and a reaction lacking any cDNA template.

H. Analysis of amplified PCR products

PCR amplified fragments containing sequences of CP and TGB1 genes were subjected to electrophoresis in 1% agarose gel (FisherBiotech New Jersey, USA) solubilized in 0.5X TBE buffer (54g of Tris, 27.5g of Boric acid and 20ml of 0.5M

EDTA in 1liter of distilled water pH 8.0) using a microwave (28). Hot gel slurry was allowed to cool down to 50^{o}C and1µl solution of ethidium bromide (FisherBiotech) was added to the 40ml solubilized gel. Gel was poured in a GT UV-transparent tray (Biorad). Tray containing solidified gel was submerged into an electrophoresis chamber (Biorad) containing 0.5X TBE electrophoresis buffer. A mixture of 6X MassRuler DNA sample loading dye (Fermentas, Canada) and PCR product 1:5 (v/v) was loaded into each of the wells. Also, 2µl of 1Kb 0' GeneRulers DNA ladder (Fermentas) was loaded in a parallel lane. Electrophoresis was done at 150 Voltage until the tracking dye (bromophenol blue) had migrated 3/4 of the gel length towards the positive electrode. The gel was then visualized and photographed under white light transilluminator universal hood (Bio-Rad) (Figures 2.6 and 2.7).

I. Purification of PCR products

Amplicons of CP and TGB1 genes were purified using MinElute PCR purification kit (Qiagen) following the manufacturer's instructions. Each of the amplified PCR products was eluted twice from each Quigen purification column. The second elute was analyzed by electrophoresis as described above. Purified PCR products containing the sequences of CP and TGB1 genes were quantified using a smart Spectrophotometer (Bio-Rad).

J. Nucleotide sequence determination and analysis

Purified PCR products were submitted to the Sequencing Laboratory Molecular Biology Resource Facility, University of Tennessee for sequencing using ABI 3730 DNA analyzer (Applied Biosystems). Sequences of both CP and TGB1 genes were determined bi-directionally, that is, nucleotide sequences were obtained from both the strands while utilizing both forward and reverse primers. Nucleotide sequences obtained were edited and assembled with vector NTI software (Invitrogen). The consensus sequences obtained represent the CP or TGB1 nucleotide sequences.

Amino acid sequences of CP and TGB1 were deduced from the generated nucleotide sequences using vector NTI software. The nucleotide and amino acid sequences of CP and TGB1 of HVX TN isolates were compared with those of HVX CP and TGB1 sequences obtained from the GenBank using Vector NTI software. Nucleotide and amino acid sequence percentage identity among isolates were determined (Tables 2.6 - 2.10).

K. Phylogenetic analysis

Sequences of HVX TN isolates obtained in this study and those of selected species representing each genus within the *Flexiviridae* family obtained from the GenBank were used for inferring phylogenies. Table 2.11 shows the members of *Flexiviridae* family that were used for phylogenetic analysis and their accession numbers as appeared in the GenBank.

Sequence alignments

Alignment of CP and TGB nucleotides and amino acids sequences was performed using Clustal X (31).

Parsimony analyses

Phylogenetic analyses of separate and combined CP and TGB nucleotides and amino acids using the parsimony criterion implemented in PAUP* 4.0b10 (29) were conducted. All characters were treated as unordered. Tree searches were conducted heuristically with tree bisection-reconnection (TBR) branch swapping in a random stepwise addition of taxa repeated 1000 times. Maxtrees was set to increase incrementally. Node support was evaluated by nonparametric bootstrap resampling (9) using neighbor-joining criteria as implemented in PAUP. Bootstrap scores were calculated from 1,000 replicates, with each replicate consisting of a single search starting with a tree built by stepwise addition using the simple addition sequence.

Bayesian analyses

Bayesian Markov chain Monte Carlo phylogenetic analysis was conducted on combined CP and TGB nucleotides using MrBayes 3.1 (25) using the model and parameters suggested by Modeltest. Each Markov chain in the Bayesian search was started from a random tree and run for 1 x 106 to 2 x 106 cycles, sampling every 1000th cycle from the chain. Four chains were run simultaneously, 3 hot and one cold. Each simulation was run twice. The default settings for the priors on the rate matrix (0-100), branch lengths (0-10), and proportion of invariant sites (0-1) were used. Stationarity was evaluated by monitoring likelihood values graphically. The initial 1,000 trees in each run were discarded as burn-in samples. The remaining trees were used to construct majority

rule consensus trees. Bayesian posterior probabilities for each clade were derived from the trees remaining after discarding the burn-in samples. For ease of visual comparison bootstrap values are presented these probabilities as whole numbers ranging from 0-100. Posterior probabilities greater than or equal to 95% are generally regarded as strong support for a clade's existance (35).

2.3. Results

A. HVX in TN

To establish the presence of HVX in TN, a total of 82 hosta plants of 31 different cultivars were collected from different regions and analyzed. Based on squash immunoblotting assays, 61 out of the 82 were infected with HVX. Most of the HVX–positive hostas showed pronounced symptoms including puckering, distortion, mosaic, chlorosis and necrosis of the foliage (Figure 2.1). SDS-PAGE analysis of HVX CP with extract from HVX–infected leaf tissues showed a protein band of 23KDa (Figure 2.3A). The nature of this protein as CP of HVX was confirmed with western immunoblotting assay (Figure 2.3B). *N. benthamiana*, infected with sap from HVX–infected leaf developed symptoms included leaf deformation, puckering and discoloration (Figure 2.4). These plants were tested HVX–positive by squash immunoblotting assay 14 days post-inoculation (Figure 2.5).

Nucleic acid analysis

The CP gene of 30 HVX–isolates from selected hosta cultivars were amplified by RT–PCR. All selected HVX–infected hostas produced positive result in RT–PCR assays (Figure 2.6; lane 3-32) confirming the serological and biological analyses. The amplified

PCR fragments were 850bp in size. No PCR products were amplified when RNA from HVX-free hosta served as templates in RT-PCR reactions (Figure 2.6; lane2) or PCR done in the absence of any template (Figure 2.6; lane1).

B. Molecular variation of HVX isolates

The genetic variability of HVX was analyzed based on the nucleotide and amino acid sequences of TGB1 and CP genes generated from 30 different TN isolates, and other isolates present in the GenBank.

Generation of sequences and their comparison

The CP gene from all TN isolates had a length of 663 nucleotides encoding a protein of 220 amino acids. CP sequences of TN isolates were compared with sequences of HVX-Kr and another US isolates, which are currently the only available HVX CP sequences in the GenBank (Tables 2.6 and 2.7). Multiple sequence alignments of all HVX CP sequences, including the Kr and US isolates were fully collinear without gaps. The alignment showed sequence conservation which was actually greater when respective amino acid sequences were used (Figures 2.8-2.11). This is probably so because there is more pressure for retaining protein structure due to its multiple functional roles in virus life cycle. A total of 42 nucleotide substitutions were found among CP of all the isolates sequenced. Seven amino acid substitutions were found among CP sequences of all the isolates when compared. These occurred in only 6 isolates: Mp-9, Mp-5, Na-5, Na-8, Jn-1 and Mp-3. The changes were unevenly distributed along the CP sequence; the 3' proximal of the CP gene is the least variable.

For the purpose of this report the first 15 amino acids (1-15) are referred to as the 5' proximal sequences, amino acids at the position 16-205 are considered as the middle part while the last 15 amino acids (206-220) are referred to as the 3' proximal sequences of the CP gene. There are 3, 3 and 1 amino acid changes at the 5' proximal, middle and 3' proximal of CP regions, respectively. One could presume that the 3' proximal of CP gene is more conserved due to its role in accomplishing the essential function of the gene. For example, CP of PVX is essential in the virus infection cycle (34). It plays a key role in the accumulation of genomic RNA, virion assembly, cell-to-cell and long distance movements, and symptoms development (3, 8, 17). CP is also important for genome encapsidation, virion morphology and viral pathogenecity (3, 33). It has been experimentally shown that infectious clone of PVX with mutation at 3' end of CP was unable to accumulate in the inoculated and systemic leaves (3). Zayakina *et al* (40) supported this finding by demonstrating that 10 amino acids at 3' proximal of PVX CP is the site recognized by TGB1 protein and this is essential for the TGBp1-triggered activation of PVX translation. They further showed that deletion of 18 amino acids at the 3' end of PVX CP led to inhibition of cell-to-cell movement of the virus and presumed that this could be due to inability of TGB1 protein to interact with CP *in vivo*.

The analyses showed that CP sequences of isolates from different geographical regions in TN shared 98.3–100% nucleotide sequence identity and 98.6–100% amino acid sequence identity. When CP sequences of TN isolates were compared with that of HVX-Kr isolate (accession number AJ620114), sequence identity at nucleotide level ranged from 98.8–99.7% and 99.1–100% at amino acid level. TN and the US isolates

(HVX-US) (accession number AJ517352) shared 98.5–99.5% nucleotide sequence identity and 99.1-100% amino acid sequence identity.

TGB1 sequences of TN isolates have a length of 696 nucleotides and are 3 nucleotides longer than that of TGB1 from HVX-Kr isolate, which is currently the only HVX TGB1 sequence available in the GenBank. Hence, the alignments of TGB1 sequences of HVX-TN isolates with that of HVX-Kr isolate have 3 gaps at position 264, 265 and 273 corresponding to nucleotides GCC except for the isolate Na–9 which corresponds to ACC when compared with the HVX-Kr isolate. TGB1 proteins from all the HVX-TN isolates are 231 amino acids in length, which are one amino acid longer than TGB1 protein of the HVX-Kr isolate. This missing amino acid is proline at position 89. Analysis showed that TGB1 sequences of HVX-TN isolates shared 98.1–100% sequence identity at nucleotide level and 98.3–100% at amino acid level among each other. When compared with the TGB1 HVX-Kr isolate, sequence identity falls between 97.4–99.1% at nucleotide level and 97–98.3% at amino acid level (Table 2.8 and 2.9).

TGB1 region of HVX isolates appear to be more variable than the CP region having 3% divergence compared to CP with 1.7% divergence (Table 2.10). In addition, 11 amino acid changes that were found among the TGB1 sequences occurred in 16 different isolates. This is in contrast to only 7 amino acid substitutions that occurred among CP sequences. One could suggest that CP is less variable because of its pivotal roles in the virus life cycle as mentioned above. The reason for more variability in TGB1 as compared to CP remains to be understood.

Phylogenetic Analysis

Phylogenetic relationships among HVX isolates and with selected species from the family *Flexivirdae* were inferred for two regions within the virus genome. However, the outcome differed slightly based on the region considered for the comparison. (Figures 2.8-2.11).

Phylogenetic analysis of the amino acid sequences of the CP gene revealed a single group of HVX isolates closely related to *Plantago asiatica mosaic virus* (PIAMV), *Tulip virus X* (TVX) and *Cassava common mosaic virus* (CsCMV) (Figure 2.8) as previously reported by Park and Ryu (22). Phylogenetic analysis of TGB1 amino acid sequences generated two groups of HVX isolates whose separation had no geographical relationship. The analysis revealed *Papaya mosaic virus* (PapMV), *Alternanthera mosaic virus* (AltMV), *Cactus virus X* (CVX), TVX and PIAMV as the sister group (Figure 2.9). To achieve better insights into the phylogenetic relationships among HVX isolates and with other species of the *Flexivirdae* family, the sequences of the TGB1 and CP genes were combined and analyzed. Phylogenetic analysis of the amino acid sequences of TGB1 and CP combined grouped HVX into two clusters (Figure 2.10). Both the HVX-Kr and the HVX-US isolates fall within one of these groups. The grouping did not show any geographical correlation nor was influenced by the type of cultivars from which the HVX isolates were obtained. Analysis also revealed HVX to be closely related to PIAMV, TVX and CsCMV. Interestingly, similar HVX groupings were observed when amino acid sequences of TGB1 gene alone were used for the analysis. This could be evidence of two distinct HVX strains. Nucleotide analysis of TGB1 and CP combined grouped HVX isolates into three distinct clusters (Figure 2.11) which however, have no correlation with

either geographical origin or host cultivars. Nucleotide sequences of TGB1 and CP genes were not analyzed phylogenetically due to extreme divergence among taxa, making alignment very tenuous.

2.4. Discussion

The widespread occurrence of HVX in TN was established based on the high rate of HVX infection in hostas collected from different geographical regions of the state. Seventy-four percent of the collected hosta plants, 61 out of 82, were infected with HVX. All the isolates, irrespective of the location from which they were collected or cultivars from which they were isolated, shared close sequence similarity. Sequence comparison of TGB1 and CP genes with previously published HVX isolates revealed close relation among TN isolates as well as with the HVX-Kr and HVX-US isolates. This suggests a common origin for all these HVX isolates included in this study.

Genetic variability of HVX was analyzed based on the sequences of TGB1 and CP genes. The results of analyses of sequences derived from 30 TN isolates along with HVX isolates obtained from GenBank revealed that the CP gene is less variable compared to the TGB1, suggesting that CP could be more under selection pressure than is TGB1 and hence, it is less variable. Nucleotide sequences of TGB1 from all the TN isolates studied here were 3 nucleotides longer than that of the Kr isolate which translated into a single amino acid insertion event. However, variation observed in both genes is relatively low, consisting of only a few base substitutions which could be due to the error-prone replication process of the virus genome. Most of the base substitutions are synonymous, but there are a few non-synonymous substitutions as well. Overall, HVX

can be considered genetically homogeneous. The genetic structure of different HVX isolates varied randomly without correlation with the geographical location or host cultivars.

As an RNA virus, HVX is expected to be prone to variation due to lack of proof-reading ability of RNA-dependant RNA polymerase (RdRp). RdRp introduces mutation in plant viruses at a rate of one substitution per genome per replication cycle (7). However, low genetic variability has been previously reported within the *Flexiviridae*, such as in C*ymbidium mosaic virus* (CymMV) (5). Members of related families such as C*losteroviridae*, have also been reported to show high genetic homogeneity, despite the fact they are transmitted by vectors. For example, isolates of *Sweet potato chlorotic stunt virus* from different countries in East and West Africa show high genetic homogeneity in their CP sequences (1). Other RNA viruses reported to exhibit low genetic variation include *Potato leafroll virus* (PLRV) isolates, *Luteoviridae*, obtained from different parts of the world (11).

Low genetic variability of HVX CP sequence could be attributed to its very narrow host range, as hosta is the only natural host of the virus. It also experimentally infects only *N. benthamiana* (15). Similarly, PLRV isolates had been reported to naturally infect only a few species of potato, which might have imposed constraints on the genetic variability of PLRV. In contrasts, isolates of *Barley yellow dwarf virus*-PAV (BYDV-PAV) require different hosts to complete their epidemiological cycle and are more genetically variable. Moreover, genetic homogeneity of HVX could be related to absence of any insect-vector as a means of transmission. BYDV-PAV is transmitted by different aphid species between different hosts and exhibits high genetic variation (11).

Absence of an insect-vector for transmission along with a very narrow host range subjects HVX to less genetic variability.

Nonetheless, single amino acid changes in CP of most plant viruses are highly significant for the virus-host interaction. The CP of several viruses is often responsible for inducing variable symptoms in plant hosts (12). A single amino acid change in the CP of *Cucumber mosaic virus* is responsible for symptom expression and virus localization in infected tobacco (30). Likewise, few amino acids of TGB1 contain determinants of viral pathogenicity in PVX (12). Future research may reveal the biological significance of the discovered amino acid substitutions in CP and TGB1 of the isolates of HVX studied here.

Moreover, the taxonomic position of HVX as a member of the *Flexiviridae* as determined in this study is in full agreement with a previous report determined solely based on sequences of HVX-Kr isolate. Park and Ryu (22) showed that HVX is closely related to PapMV, AltMV and CVX based on nucleotide percentage identity of CP from the HVX-Kr isolate. However with phylogenetic analysis of 2.7 kb nucleotide sequences of the genome, they reported that HVX is more related to PIAMV, TVX and CsCMV. The same conclusion can be drawn from this research where phylogenetic analysis was done with combined amino acid sequences of CP and TGB1, which showed that PapMV, AltMV, CVX, PIAMV, TVX and CsCMV cluster together and form a sister group to HVX. Furthermore, phylogenetic analysis revealed that most variations observed among isolates of HVX are unrelated, that is, not shared among all the isolates. Two groups observed in the reconstructed phylogenetic tree from combined TGB1 and CP amino acid sequences are not supported with significant bootstrap value. Likewise, phylogenetic

analysis of nucleotide sequences of CP and TGB1 combined revealed three groups all with bootstrap values less than 90. In addition, phylogenetic analysis of amino acid sequences of TGB1 alone generated two groups with only a 78% bootstrap score while the analysis with CP amino acid sequence alone showed no grouping of HVX isolates. These results suggest that relationships among HVX isolates are unresolved.

From a practical standpoint, however, the low sequence diversity of HVX CP gene could be of an advantage in regard to the virus diagnostics and control. A conserved sequence of HVX CP is important in serological based diagnosis assay as antibodies could be developed against such conserved region that will recognize most or all of the HVX isolates. Another practical implication of low variability among CP sequences of HVX is for the development of engineered resistance against the virus in hosta. CP-mediated resistance strategy has been utilized for control of viral diseases of plants; however, its success depends on the sequence similarity between the transgenes and the virus infecting the plants (5). CymMV a member of *Potexvirus* and *Odontoglossum ringspot virus* a member of *Tobamovirus* have been reported to have high conserved CP and this made CP gene suitable candidate to provide resistance to their host plant, orchids (5). However, there are over 2,500 hosta cultivars and application of CP-mediated resistance to all is not practically feasible. Conserved sequences of HVX CP can be also useful in development of nucleotide based diagnostic assay. For example, primers could be generated within the conserved region of the gene to amplify all HVX isolates by RT-PCR alone or in combination with restriction digestion analysis developed for many other RNA viruses (23, 37, 38).

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Appendix 2: Tables

Table 2.1 Hosta plants exhibiting HVX symptoms collected from different regions of Tennessee

Region	**Location**	**No. of cultivars**	**No. of samples**	**No. positive in squash blot**	**No. sequenced**
EastTennessee	Knoxville	8	17	12	3
	Morristown	4	6	2	2
	Johnson	1	1	1	1
	Greenville	1	3	3	1
Middle Tennessee	Nashville	6	9	7	5
	Murfreesboro	6	11	5	2
	Franklin	4	4	4	3
	Smyrna	1	1	1	1
WestTennessee	Jackson	7	20	16	5
	Memphis	9	10	10	7
Total		31	82	61	30

Table 2.2 Hosta cultivars, locations and diagnosis

#	Isolates	Cultivar	Location	Squash Blot	Agdia strip	RT-PCR
1	TnKn-1	Blue Cadet	Knoxville	+	ND	ND
2	TnKn-2	Bressingham Blue	Knoxville	+	ND	+
3	TnKn-3	Frances Williams	Knoxville	-	ND	ND
4	TnKn-4	Blue Cadet	Knoxville	+	ND	ND
5	TnKn-5	Blue Cadet	Knoxville	+	ND	ND
6	TnKn-6	Blue Cadet	Knoxville	+	ND	ND
7	TnKn-7	Blue Cadet	Knoxville	+	ND	ND
8	TnKn-8	Blue Cadet	Knoxville	+	ND	ND
9	TnKn-9	Karin	Knoxville	+	ND	ND
10	TnKn-10	Hardspen Lavender	Knoxville	-	ND	ND
11	TnKn-11	Frances Williams	Knoxville	+	ND	ND
12	TnKn-12	Blue Cadet	Knoxville	+	ND	ND
13	TnKn-13	Minuteman	Knoxville	-	ND	ND
14	TnKn-14	Blue Cadet	Knoxville	+	ND	+

Table 2.2 Continued

#	Isolates	Cultivar	Location	Squash Blot	Agdia strip	RT-PCR
15	TnKn-15	Christmas Tree	Knoxville	-	ND	ND
16	TnKn-16	Mediovariegata	Knoxville	+	ND	+
17	TnKn-17	Albo Marginata	Knoxville	-	ND	ND
18	TnMt-1	Moorheim	Morristown	-	ND	ND
19	TnMt-2	Moorheim	Morristown	-	ND	ND
20	TnMt-3	Gold Standard	Morristown	-	ND	ND
21	TnMt-4	Medio Variegata	Morristown	-	ND	ND
22	TnMt-5	Austin Dickinson	Morristown	+	ND	+
23	TnMt-6	Gold Standard	Morristown	+	ND	+
24	TnJn-1	August Moon	Johnson	+	+	+
25	TnGn-1	Golden Tiara	Greenville	+	ND	ND
26	TnGn-2	Golden Tiara	Greenville	+	ND	ND
27	TnGn-3	Golden Tiara	Greenville	+	ND	+
28	TnNa-1	Aureo Marginata	Nashville	+	+	+

Table 2.2 Continued

#	Isolates	Cultivar	Location	Squash Blot	Agdia strip	RT-PCR
29	TnNa-2	Albo Marginata	Nashville	+	+	ND
30	TnNa-3	Albo Marginata	Nashville	+	+	ND
31	TnNa-4	Medio Variegata	Nashville	-	+	ND
32	TnNa-5	Yellow Splash	Nashville	+	+	+
33	TnNa-6	Antioch	Nashville	+	+	+
34	TnNa-7	Albo Marginata	Nashville	-	-	ND
35	TnNa-8	Royal Standard	Nashville	+	+	+
36	TnNa-9	Albo-Marginata	Nashville	+	+	+
37	TnMf-1	Sum and Substance	Murfreesboro	-	ND	ND
38	TnMf-2	Sum and Substance	Murfreesboro	-	ND	ND
39	TnMf-3	Sum and Substance	Murfreesboro	-	ND	ND

Table 2.2 Continued

#	Isolates	Cultivar	Location	Squash Blot	Agdia strip	RT-PCR
40	TnMf-4	Gold Standard	Murfreesboro	+	+	ND
41	TnMf-5	Gold Standard	Murfreesboro	+	+	+
42	TnMf-6	Frances Williams	Murfreesboro	-	-	ND
43	TnMf-7	Fragrant Blue	Murfreesboro	-	-	ND
44	TnMf-8	Albo Marginata	Murfreesboro	-	-	ND
45	TnMf-9	Golden Tiara	Murfreesboro	+	+	ND
46	TnMf-10	Golden Tiara	Murfreesboro	+	+	+
47	TnMf-11	Golden Tiara	Murfreesboro	+	+	ND
48	TnFr-1	Lady Guinevere	Franklin	+	+	ND

Table 2.2 Continued

#	Isolates	Cultivar	Location	Squash Blot	Agdia strip	RT-PCR
49	TnFr-2	August Moon	Franklin	+	+	+
50	TnFr-3	Pilgrim	Franklin	+	+	+
51	TnFr-4	Sum and Substance	Franklin	+	+	+
52	TnSm	August Moon	Smyrna home depot	+	+	+
53	TnJa-1	Christmas Tree	Jackson	-	ND	ND
54	TnJa-2	Christmas Tree	Jackson	-	ND	ND
55	TnJa-3	Sagae	Jackson	-	ND	ND
56	TnJa-4	Lady Guinevere	Jackson	+	ND	ND
57	TnJa-5	Lady Guinevere	Jackson	+	ND	ND
58	TnJa-6	Lady Guinevere	Jackson	+	ND	ND

Table 2.2 Continued

#	Isolates	Cultivar	Location	Squash Blot	Agdia strip	RT-PCR
59	TnJa-7	Lady Guinevere	Jackson	+	ND	+
60	TnJa-8	Lady Guinevere	Jackson	+	ND	+
61	TnJa-9	Lady Guinevere	Jackson	-	ND	ND
62	TnJa-10	Lady Guinevere	Jackson	+	ND	ND
63	TnJa-11	Revolution	Jackson	+	+	+
64	TnJa-12	Sum and substance	Jackson	+	+	ND
65	TnJa-13	Sum and Substance	Jackson	+	+	+
66	TnJa-14	August Moon	Jackson	+	+	+
67	TnJa-15	Revolution	Jackson	+	+	ND
68	TnJa-16	Wide Brim	Jackson	+	+	+
69	TnJa-17	Revolution	Jackson	+	+	ND

Table 2.2 Continued

#	Isolates	Cultivar	Location	Squash Blot	Agdia strip	RT-PCR
70	TnJa-18	Revolution	Jackson	+	+	ND
71	TnJa-19	Revolution	Jackson	+	+	ND
72	TnJa-20	Sum and Substance	Jackson	+	+	ND
73	TnMp-1	Lancifolia	Memphis	+	+	+
74	TnMp-2	Blue Cadet	Memphis	+	+	+
75	TnMp-3	So Sweet	Memphis	+	+	+
76	TnMp-4	Golden Tiara	Memphis	+	+	+
77	TnMp-5	Gold Standard	Memphis	+	+	+
78	TnMp-6	Scooter	Memphis	+	+	ND
79	TnMp-7	Spritzer	Memphis	+	+	+
80	TnMp-8	Gold Standard	Memphis	+	+	ND
81	TnMp-9	Blue Diamond	Memphis	+	+	+
82	TnMp-10	August Moon	Memphis	+	+	ND

Symbols indicate presence (+) or absence (-) of HVX. ND indicate the test was not done

Table 2.3 Comparison of genomic regions of *Potato virus X* isolates

	Accession numbers	**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
Replicase	**NC_001455**	X	95	95	99	100
	AB056719	95	X	96	94	95
	AB056718	95	96	X	95	95
	M38480	99	94	95	X	99
	M72416	100	95	95	99	X
TGB1		**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
	NC_001455	X	95	96	100	100
	AB056719	95	X	97	95	95
	AB056718	96	97	X	96	96
	M38480	100	95	96	X	100
	M72416	100	95	96	100	X
TGB 2		**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
	NC_001455	X	96	97	100	.
	AB056719	96	X	97	96	96
	AB056718	97	97	X	97	97
	M38480	100	96	97	X	100
	M72416	.	96	97	100	X

Table 2.3 Continued

	Accession numbers	**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
TGB 3		**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
	NC_001455	X	98	98	100	100
	AB056719	98	X	98	98	98
	AB056718	98	98	X	98	98
	M38480	100	98	98	X	100
	M72416	100	98	98	100	X
Coat Protein		**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
	NC_001455	X	95	95	100	100
	AB056719	95	X	96	95	95
	AB056718	95	96	X	95	95
	M38480	100	95	95	X	100
	M72416	100	95	95	100	X
5' End		**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
	NC_001455	X	96	96	100	100
	AB056719	96	X	96	96	96
	AB056718	96	96	X	96	96
	M38480	100	96	96	X	.
	M72416	100	96	96	.	X
	M72416	100	100	100	100	X

Table 2.3 Continued

	Accession numbers	**NC_001455**	**AB056719**	**AB056718**	**M38480**	**M72416**
3' End	**NC_001455**	X	100	100	100	100
	AB056719	100	X	.	100	100
	AB056718	100	.	X	100	100
	M38480	100	100	100	X	100
	M72416	100	100	100	100	X

Table 2.4 Primers used for first strand cDNA synthesis, PCR amplification and sequencing reactions

	Primer Name	**Sequence 5' – 3'**	**Position on HVX genome**
cDNA synthesis	HVXa6488	TTAGTGGAACGGTTAGCC C	6488 - 6470
	Random	NNNNNNNNNN	Non viral sequence
	Oligo dt	TTTTTTTTTTTTTTT	Non viral sequence
CP amplification and sequencing	HVXs5639	TTCTAGAAGGAAACTGCGCT	5639 – 5658
	HVXs5722	AGTCTCGAACTA ACT AACAGG	5722 – 5742
	HVXs6014 (internal primer)	GCCAGGCACAAC GGTCAACTAC	6014 – 6035
	HVXa6169 (internal primer)	TCTTTGTAGCCCATCGCCGC	6169 – 6149
	HVXa6448	TCGGTGGAGCCTTGTTTATTG	6448 – 6428
	HVXa6488	TTAGTGGAACGGTTAGCC C	6488 – 6470
	HVXa6499	CACACTTTAAGTTAGTGGAACGG	6499 – 6477
TGB1 amplification and sequencing	HVXs4501	CAGTGCAGAACCCACCAAGT	4501 – 4520
	HVXs4892 (internal)	CTCCAACACTACGAGAGGGC	4892 – 4911
	HVXa4995 (internal)	ATGTCGAGGCCGAGCTTTCT	4995 – 4976
	HVXa5335	ACTACCGCTAGTCCTACTCCTAT	5335 – 5313
	HVXa5361	GCG TTG ACC TAG TTA GCT GG	53461 – 5342
	HVXa5365	GGA AGC GTT GAC CTA GTT AG	5365 – 5346
	HVXa5377	TCT CCA ACG TGG GGA AGC GT	5377 – 5358

Sequence positions are based on the complete HVX sequences derived from Kr isolate (GenBank accession number AJ620114).

Table 2.5 Origins of HVX isolates subjected to sequencing and GenBank accession numbers

#	Isolates	CP GenBank accession no.	TGB1 GenBank accession no.	Source (Hosta cultivar)	Location
1	TnKn-2	FJ903387	FJ903428	Bressingham Blue	Knoxville
2	TnKn-14	FJ903393	FJ903421	Blue Cadet	Knoxville
3	TnKn-16	FJ903394	FJ903422	Medio-variegata	Knoxville
4	TnMt-5	FJ903396	FJ903425	Austin Dickinson	Morristown
5	TnMt-6	FJ903399	FJ903429	Gold Standard	Morristown
6	TnJn-1	FJ903386	FJ903420	August Moon	Johnson
7	TnGn-3	FJ903395	FJ903424	Golden Tiara	Greenville
8	TnNa-1	FJ903389	FJ903417	Aureo Marginata	Nashville
9	TnNa-5	FJ903398	FJ903427	Yellow Splash	Nashville
10	TnNa-6	FJ903390	FJ903423	Antioch	Nashville
11	TnNa-8	FJ903401	FJ903431	Royal Standard	Nashville
12	TnNa-9	FJ903402	FJ903432	Albo-Marginata	Nashville
13	TnMf-5	FJ903397	FJ903426	Gold Standard	Murfreesboro
14	TnMf-10	FJ903391	FJ903418	Gold Standard	Murfreesboro
15	TnFr-2	FJ903403	FJ903433	August Moon	Franklin
16	TnFr-3	FJ903392	FJ903419	Pilgrim	Franklin
17	TnFr-4	FJ903400	FJ903430	Sum and Substance	Franklin
18	TnSm-1	FJ903415	FJ903445	August Moon	Smyrna

Table 2.5 Continued

#	Isolates	CP GenBank accession no.	TGB1 GenBank accession no.	Source (Hosta cultivar)	Location
19	TnJa-7	FJ903388	FJ903416	Lady Guinevere	Jackson
20	TnJa-11	FJ903405	FJ903435	Revolution	Jackson
21	TnJa-13	FJ903409	FJ903439	Sum and Substance	Jackson
22	TnJa-14	FJ903410	FJ903440	August Moon	Jackson
23	TnJa-16	FJ903407	FJ903437	Wide Brim	Jackson
24	TnMp-1	FJ903404	FJ903434	Lancifolia	Memphis
25	TnMp-2	FJ903408	FJ903438	Blue Cadet	Memphis
26	TnMp-3	FJ903406	FJ903436	So Sweet	Memphis
27	TnMp-4	FJ903412	FJ903442	Golden Tiara	Memphis
28	TnMp-5	FJ903413	FJ903443	Gold Standard	Memphis
29	TnMp-7	FJ903411	FJ903441	Spritzer	Memphis
30	TnMp-9	FJ903414	FJ903444	Blue Diamond	Memphis

Table 2.6 Percentage amino acid identity of CP gene among all HVX isolates

	Kr	U.S	Kn-2	Ja-7	Mf-5	Na-1	Na-5	Na-6	Mf-10	Fr-2	Fr-3	Jn-1	Kn-14	Kn-16	Gn-3	Mt-5	Mt-6	Fr-4	Na-8	Na-9	Mp-1	Ja-11	Mp-2	Ja-13	Mp-3	Ja-14	Mp-4	Mp-5	Ja-16	Mp-7	Mp-9	Sm-1
Kr	X	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
U.S	100	X	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Kn-2	100	100	X	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Ja-7	100	100	100	X	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Mf-5	100	100	100	100	X	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Na-1	100	100	100	100	100	X	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Na-5	99.1	99.1	99.1	99.1	99.1	99.1	X	99.1	99.1	99.1	99.1	98.6	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	98.6	99.1	99.1	98.6	99.1	99.1	98.6	99.1
Na-6	100	100	100	100	100	100	99.1	X	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Mf-10	100	100	100	100	100	100	99.1	100	X	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Fr-2	100	100	100	100	100	100	99.1	100	100	X	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Fr-3	100	100	100	100	100	100	99.1	100	100	100	X	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Jn-1	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	X	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	99.5	99.1	99.5	99.5	99.1	99.5	99.5	99.1	99.5
Kn-14	100	100	100	100	100	100	99.1	100	100	100	100	99.5	X	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Kn-16	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	X	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Gn-3	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	X	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Mt-5	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	X	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Mt-6	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	X	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Fr-4	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	X	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Na-8	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	99.1	98.6	99.1	99.1	99.1	99.1	99.1	99.1	X	99.1	99.1	99.1	99.1	99.1	98.6	99.1	99.1	98.6	99.1	99.1	98.6	99.1
Na-9	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	X	100	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Mp-1	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	X	100	100	100	99.5	100	100	99.5	100	100	99.5	100
Ja-11	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	X	100	100	99.5	100	100	99.5	100	100	99.5	100
Mp-2	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	X	100	99.5	100	100	99.5	100	100	99.5	100
Ja-13	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	X	99.5	100	100	99.5	100	100	99.5	100
Mp-3	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	99.1	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	99.5	X	99.5	99.5	99.1	99.5	99.5	99.1	99.5
Ja-14	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	X	100	99.5	100	100	99.5	100
Mp-4	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	X	99.5	100	100	99.5	100
Mp-5	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	99.1	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	99.5	99.1	99.5	99.5	X	99.5	99.5	99.1	99.5
Ja-16	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	X	100	99.5	100
Mp-7	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	X	99.5	100
Mp-9	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	99.1	99.5	99.5	99.5	99.5	99.5	99.5	98.6	99.5	99.5	99.5	99.5	99.5	99.1	99.5	99.5	99.1	99.5	99.5	X	99.5
Sm-1	100	100	100	100	100	100	99.1	100	100	100	100	99.5	100	100	100	100	100	100	99.1	100	100	100	100	100	99.5	100	100	99.5	100	100	99.5	X

Table 2.7 Percentage nucleotide identity of CP gene among all HVX isolates

	Kr	U.S	Kn-2	Ja-7	Mf-5	Na-1	Na-5	Na-6	Mf-10	Fr-2	Fr-3	Jn-1	Kn-14	Kn-16	Gn-3	Mt-5	Mt-6	Fr-4	Na-8	Na-9	Mp-1	Ja-11	Mp-2	Ja-13	Mp-3	Ja-14	Mp-4	Mp-5	Ja-16	Mp-7	Mp-9	Sm-1
Kr	X	98.9	99.5	99.1	99.5	99.4	99.2	99.4	99.2	99.5	99.5	98.9	99.2	99.5	99.5	99.4	99.4	99.4	99.2	99.5	98.8	99.7	99.2	99.4	99.4	99.2	99.2	99.1	99.4	99.2	99.4	99.5
U.S	98.9	X	99.1	98.6	99.4	99.2	98.8	99.2	99.1	99.1	99.1	98.5	99.1	99.1	99.4	99.2	99.5	99.2	98.6	99.1	98.5	99.2	99.1	98.9	98.9	98.8	98.8	98.9	99.2	98.8	98.9	99.4
Kn-2	99.5	99.1	X	99.5	99.7	99.5	99.4	99.5	99.4	100	98.5	99.4	99.4	100	99.7	99.5	99.5	99.5	99.2	99.7	98.9	99.8	99.4	99.8	99.8	99.7	99.7	99.2	99.8	99.7	99.5	99.7
Ja-7	99.1	98.6	99.5	X	99.2	99.1	98.9	99.1	98.9	99.5	99.5	99.2	98.9	99.5	99.2	99.1	99.1	99.1	98.8	99.2	98.5	99.4	98.9	99.4	99.4	99.2	99.2	98.8	99.4	99.2	99.1	99.2
Mf-5	99.5	99.4	99.7	99.2	X	99.8	99.4	99.8	99.7	99.7	99.7	99.1	99.7	99.7	100	99.8	99.8	99.8	99.2	99.7	98.9	99.8	99.7	99.5	99.5	99.4	99.4	99.5	99.5	99.4	99.5	100
Na-1	99.4	99.2	99.5	99.1	99.8	X	99.2	99.7	99.5	99.5	99.5	98.9	99.5	99.5	99.8	99.7	99.7	99.7	99.1	99.5	98.8	99.7	99.5	99.4	99.4	99.2	99.2	99.4	99.4	99.2	99.4	99.8
Na-5	99.2	98.8	99.4	98.9	99.4	99.2	X	99.2	99.1	99.4	99.4	98.8	99.1	99.4	99.4	99.2	99.2	99.2	99.5	99.4	98.6	99.5	99.1	99.2	99.2	99.1	99.1	98.9	99.2	99.1	99.2	99.4
Na-6	99.4	99.2	99.5	99.1	99.8	99.7	99.2	X	99.5	99.5	99.5	98.9	99.8	99.5	99.8	99.7	99.7	99.7	99.1	99.5	98.8	99.7	99.5	99.4	99.4	99.2	99.2	99.4	99.4	99.2	99.4	99.8
Mf-10	99.2	99.1	99.4	98.9	99.7	99.5	99.1	99.5	X	99.4	99.4	98.8	99.4	99.4	99.7	99.5	99.5	99.5	98.9	99.4	98.6	99.5	99.4	99.2	99.2	99.1	99.1	99.2	99.2	99.1	99.2	99.7
Fr-2	99.5	99.1	100	99.5	99.7	99.5	99.4	99.5	99.4	X	100	99.4	99.4	100	99.7	99.5	99.5	99.5	99.2	99.7	98.9	99.8	99.4	99.8	99.8	99.7	99.7	99.2	99.8	99.7	99.5	99.7
Fr-3	99.5	99.1	100	99.5	99.7	99.5	99.4	99.5	99.4	100	X	99.4	99.4	100	99.7	99.5	99.5	99.5	99.2	99.7	98.9	99.8	99.4	99.8	99.8	99.7	99.7	99.2	99.8	99.7	99.5	99.7
Jn-1	98.9	98.5	99.4	99.2	99.1	98.9	98.8	98.9	98.8	99.4	99.4	X	98.8	99.4	99.1	98.9	98.9	98.9	98.6	99.1	98.3	99.2	98.8	99.2	99.2	99.1	99.1	98.6	99.2	99.1	98.9	99.1
Kn-14	99.2	99.1	99.4	98.9	99.7	99.5	99.1	99.8	99.4	99.4	99.4	98.8	X	99.4	99.7	99.5	99.5	99.5	98.9	99.4	98.6	99.5	99.4	99.2	99.2	99.1	99.1	99.2	99.2	99.1	99.2	99.7
Kn-16	99.5	99.1	100	99.5	99.7	99.5	99.4	99.5	99.4	100	100	99.4	99.4	X	99.7	99.5	99.5	99.5	99.2	99.7	98.9	99.8	99.4	99.8	99.8	99.7	99.7	99.2	99.8	99.7	99.5	99.7
Gn-3	99.5	99.4	99.7	99.2	100	99.8	99.4	99.8	99.7	99.7	99.7	99.1	99.7	99.7	X	99.8	99.8	99.8	99.2	99.7	98.9	99.8	99.7	99.5	99.5	99.4	99.4	99.5	99.5	99.4	99.5	100
Mt-5	99.4	99.2	99.5	99.1	99.8	99.7	99.2	99.7	99.5	99.5	99.5	98.9	99.5	99.5	99.8	X	99.7	99.7	99.1	99.5	98.8	99.7	99.5	99.4	99.4	99.2	99.2	99.4	99.4	99.2	99.4	99.8
Mt-6	99.4	99.5	99.5	99.1	99.8	99.7	99.2	99.7	99.5	99.5	99.5	98.9	99.5	99.5	99.8	99.7	X	99.7	99.1	99.5	98.8	99.7	99.5	99.4	99.4	99.2	99.2	99.4	99.4	99.2	99.4	99.8
Fr-4	99.4	99.2	99.5	99.1	99.8	99.7	99.2	99.7	99.5	99.5	99.5	98.9	99.5	99.5	99.8	99.7	99.7	X	99.1	99.5	98.8	99.7	99.5	99.4	99.4	99.2	99.2	99.4	99.4	99.2	99.4	99.8
Na-8	99.2	98.6	99.2	98.8	99.2	99.1	99.5	99.1	98.9	99.2	99.2	98.6	98.9	99.2	99.2	99.1	99.1	99.1	X	99.2	98.5	99.4	98.9	99.1	99.1	98.9	98.9	98.8	99.1	98.9	99.1	99.2
Na-9	99.5	99.1	99.7	99.2	99.7	99.5	99.4	99.5	99.4	99.7	99.7	99.1	99.4	99.7	99.7	99.5	99.5	99.5	99.2	X	98.9	99.8	99.4	99.5	99.5	99.4	99.4	99.2	99.5	99.4	99.5	99.7
Mp-1	98.8	98.5	98.9	98.5	98.9	98.8	98.6	98.8	98.6	98.9	98.9	98.3	98.6	98.9	98.9	98.8	98.8	98.8	98.5	98.9	X	99.1	98.6	98.8	98.8	98.6	98.6	98.5	98.9	98.6	98.8	98.9
Ja-11	99.7	99.2	99.8	99.4	99.8	99.7	99.5	99.7	99.5	99.8	99.8	99.2	99.5	99.8	99.8	99.7	99.7	99.7	99.4	99.8	99.1	X	99.5	99.7	99.7	99.5	99.5	99.4	99.7	99.5	99.7	99.8
Mp-2	99.2	99.1	99.4	98.9	99.7	99.5	99.1	99.5	99.4	99.4	99.4	98.8	99.4	99.4	99.7	99.5	99.5	99.5	98.9	99.4	98.6	99.5	X	99.2	99.2	99.1	99.1	99.2	99.2	99.1	99.2	99.7
Ja-13	99.4	98.9	99.8	99.4	99.5	99.4	99.2	99.4	99.2	99.8	99.8	99.2	99.2	99.8	99.5	99.4	99.4	99.4	99.1	99.5	98.8	99.7	99.2	X	99.7	99.5	99.5	99.1	99.7	99.8	99.4	99.5
Mp-3	99.4	98.9	99.8	99.4	99.5	99.4	99.2	99.4	99.2	99.8	99.8	99.2	99.2	99.8	99.5	99.4	99.4	99.4	99.1	99.5	98.8	99.7	99.2	99.7	X	99.5	99.5	99.1	99.7	99.8	99.4	99.5
Ja-14	99.2	98.8	99.7	99.2	99.4	99.2	99.1	99.2	99.1	99.7	99.7	99.1	99.1	99.7	99.4	99.2	99.2	99.2	98.9	99.4	98.6	99.5	99.1	99.5	99.5	X	99.4	98.9	99.5	99.4	99.5	99.4
Mp-4	99.2	98.8	99.7	99.2	99.4	99.2	99.1	99.2	99.1	99.7	99.7	99.1	99.1	99.7	99.4	99.2	99.2	99.2	98.9	99.4	98.6	99.5	99.1	99.5	99.5	99.4	X	98.9	99.5	99.4	99.2	99.4
Mp-5	99.1	98.9	99.2	98.8	99.5	99.4	98.9	99.4	99.2	99.2	99.2	98.6	99.2	99.2	99.5	99.4	99.4	99.4	98.8	99.2	98.5	99.4	99.2	99.1	99.1	98.9	98.9	X	99.1	98.9	99.1	99.5
Ja-16	99.4	99.2	99.8	99.4	99.5	99.4	99.2	99.4	99.2	99.8	99.8	99.2	99.2	99.8	99.5	99.4	99.4	99.4	99.1	99.5	98.9	99.7	99.2	99.7	99.7	99.5	99.5	99.1	X	99.5	99.4	99.5
Mp-7	99.2	98.8	99.7	99.2	99.4	99.2	99.1	99.2	99.1	99.7	99.7	99.1	99.1	99.7	99.4	99.2	99.2	99.2	98.9	99.4	98.6	99.5	99.1	99.8	99.8	99.4	99.4	98.9	99.5	X	99.2	99.4
Mp-9	99.4	98.9	99.5	99.1	99.5	99.4	99.2	99.4	99.2	99.5	99.5	98.9	99.2	99.5	99.5	99.4	99.4	99.4	99.1	99.5	98.8	99.7	99.2	99.4	99.4	99.5	99.2	99.1	99.4	99.2	X	99.5
Sm-1	99.5	99.4	99.7	99.2	100	99.8	99.4	99.8	99.7	99.7	99.7	99.1	99.7	99.7	100	99.8	99.8	99.8	99.2	99.7	98.9	99.8	99.7	99.5	99.5	99.4	99.4	99.5	99.5	99.4	99.5	X

Table 2.8 Percentage amino acid identity of TGB1 among all HVX isolates

	Kr	Kn-2	Ja-7	Mf-5	Na-1	Na-5	Na-6	Mf-10	Fr-2	Fr-3	Jn-1	Kn-14	Kn-16	Gn-3	Mt-5	Mt-6	Fr-4	Na-8	Na-9	Mp-1	Ja-11	Mp-2	Ja-13	Mp-3	Ja-14	Mp-4	Mp-5	Ja-16	Mp-7	Mp-9	Sm-1
Kr	X	97.4	97.4	98.3	98.3	98.3	98.3	97.8	97.4	97.4	97.4	98.3	97	98.3	98.3	98.3	97.8	98.3	98.3	98.3	98.3	98.3	97.4	97.4	97.4	97.4	98.3	97	97	97.8	98.3
KN-2	97.4	X	100	99.1	99.1	99.1	99.1	98.7	100	100	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	100	100	100	99.1	99.6	99.6	98.7	99.1
Ja-7	97.4	100	X	99.1	99.1	99.1	99.1	98.7	100	100	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	100	100	100	99.1	99.6	99.6	98.7	99.1
Mf-5	98.3	99.1	99.1	X	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Na-1	98.3	99.1	99.1	100	X	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Na-5	98.3	99.1	99.1	100	100	X	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Na-6	98.3	99.1	99.1	100	100	100	X	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Mf-10	97.8	98.7	98.7	99.6	99.6	99.6	99.6	X	98.7	98.7	98.7	99.6	98.3	99.6	99.6	99.6	99.1	99.6	99.6	99.6	99.6	99.6	98.7	98.7	98.7	98.7	99.6	98.3	98.3	99.1	99.6
Fr-2	97.4	100	100	99.1	99.1	99.1	99.1	98.7	X	100	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	100	100	100	99.1	99.6	99.6	98.7	99.1
Fr-3	97.4	100	100	99.1	99.1	99.1	99.1	98.7	100	X	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	100	100	100	99.1	99.6	99.6	98.7	99.1
Jn-1	97.4	100	100	99.1	99.1	99.1	99.1	98.7	100	100	X	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	100	100	100	99.1	99.6	99.6	98.7	99.1
Kn-14	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	X	98.7	100	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Kn-16	97	99.6	99.6	98.7	98.7	98.7	98.7	98.3	99.6	99.6	99.6	98.7	X	98.7	98.7	98.7	98.3	98.7	98.7	98.7	98.7	98.7	99.6	99.6	99.6	99.6	98.7	99.1	99.1	98.3	98.7
Gn-3	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	X	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Mt-5	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	X	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Mt-6	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	X	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Fr-4	97.8	98.7	98.7	99.6	99.6	99.6	99.6	99.1	98.7	98.7	98.7	99.6	98.3	99.6	99.6	99.6	X	99.6	99.6	99.6	99.6	99.6	98.7	98.7	98.7	98.7	99.6	98.3	98.3	99.1	99.6
Na-8	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	X	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Na-9	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	X	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Mp-1	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	X	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Ja-11	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	X	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Mp-2	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	100	X	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	100
Ja-13	97.4	100	100	99.1	99.1	99.1	99.1	98.7	100	100	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	X	100	100	100	99.1	99.6	99.6	98.7	99.1
Mp-3	97.4	100	100	99.1	99.1	99.1	99.1	98.7	100	100	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	X	100	100	99.1	99.6	99.6	98.7	99.1
Ja-14	97.4	100	100	99.1	99.1	99.1	99.1	98.7	100	100	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	100	X	100	99.1	99.6	99.6	98.7	99.1
Mp-4	97.4	100	100	99.1	99.1	99.1	99.1	98.7	100	100	100	99.1	99.6	99.1	99.1	99.1	98.7	99.1	99.1	99.1	99.1	99.1	100	100	100	X	99.1	99.6	99.6	98.7	99.1
Mp-5	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	X	98.7	98.7	99.6	100
Ja-16	97	99.6	99.6	98.7	98.7	98.7	98.7	98.3	99.6	99.6	99.6	98.7	99.1	98.7	98.7	98.7	98.3	98.7	98.7	98.7	98.7	98.7	99.6	99.6	99.6	99.6	98.7	X	99.1	98.3	98.7
Mp-7	97	99.6	99.6	98.7	98.7	98.7	98.7	98.3	99.6	99.6	99.6	98.7	99.1	98.7	98.7	98.7	98.3	98.7	98.7	98.7	98.7	98.7	99.6	99.6	99.6	99.6	98.7	99.1	X	98.3	98.7
Mp-9	97.8	98.7	98.7	99.6	99.6	99.6	99.6	99.1	98.7	98.7	98.7	99.6	98.3	99.6	99.6	99.6	99.1	99.6	99.6	99.6	99.6	99.6	98.7	98.7	98.7	98.7	99.6	98.3	98.3	X	99.6
Sm-1	98.3	99.1	99.1	100	100	100	100	99.6	99.1	99.1	99.1	100	98.7	100	100	100	99.6	100	100	100	100	100	99.1	99.1	99.1	99.1	100	98.7	98.7	99.6	X

Table 2.9 Percentage nucleotide identity of TGB1 among all HVX isolates

	Kr	Kn-2	Ja-7	Mf-5	Na-1	Na-5	Na-6	Mf-10	Fr-2	Fr-3	Jn-1	Kn-14	Kn-16	Gn-3	Mt-5	Mt-6	Fr-4	Na-8	Na-9	Mp-1	Ja-11	Mp-2	Ja-13	Mp-3	Ja-14	Mp-4	Mp-5	Ja-16	Mp-7	Mp-9	Sm-1
Kr	X	98	97.7	99	99.1	99	98.7	99	98.1	98.1	97.8	98.9	98	99.1	99.1	99.1	99	99	98.3	98	98.6	99.1	97.4	97.4	97.4	98	99	97.8	98	98.4	99
KN-2	98	X	99.1	98.7	98.9	99	98.7	98.7	99.6	99.6	99.3	98.9	99.4	98.9	98.9	98.9	98.7	99	98.4	98.9	98.6	98.9	99.7	99.6	99.4	99.4	98.7	99.3	99.4	98.4	99
Ja-7	97.7	99.1	X	98.6	98.6	98.7	98.4	98.4	99.3	99.3	99.6	98.9	99.1	98.6	98.6	98.6	98.4	98.7	98.1	98.6	98.6	98.6	100	100	100	99.1	98.4	99	99.1	98.4	98.7
Mf-5	99	98.7	98.6	X	99.9	99.7	99.4	99.7	98.9	98.9	98.6	99.6	98.7	99.9	99.9	99.9	99.7	99.7	98.9	98.7	99.3	99.9	99.1	99.1	99.1	98.7	99.7	98.6	98.7	99.1	99.7
Na-1	99.1	98.9	98.6	99.9	X	99.9	99.6	99.9	99	99	98.7	99.7	98.9	100	100	100	99.9	99.9	99	98.9	99.4	100	99.1	99.1	99.1	98.9	99.9	98.7	98.9	99.3	99.9
Na-5	99	99	98.7	99.7	99.9	X		99.7	99.1	99.1	98.9	99.6	99	99.9	99.9	99.9	99.7	100	99.1	99	99.6	99.9	99.1	99.1	99.1	99	99.7	98.9	99	99.4	99.7
Na-6	98.7	98.7	98.4	99.4	99.6	99.4	X	99.4	98.9	98.9	98.6	99.3	98.7	99.6	99.6	99.6	99.4	99.4	98.6	98.7	99	99.6	99	98.9	98.7	98.7	99.4	98.6	98.7	98.9	99.7
Mf-10	99	98.7	98.4	99.7	99.9	99.7	99.4	X	98.9	98.9	98.6	99.6	98.7	99.9	99.9	99.9	99.7	99.7	98.9	98.7	99.3	99.9	98.7	98.7	98.7	98.7	99.7	98.6	98.7	99.1	99.7
Fr-2	98.1	99.6	99.3	98.9	99	99.1	98.9	98.9	X	100	99.4	99	99.6	99	99	99	98.9	99.1	98.6	99	98.7	99	100	100	100	99.6	98.9	99.4	99.6	98.6	99.1
Fr-3	98.1	99.6	99.3	98.9	99	99.1	98.9	98.9	100	X	99.4	99	99.6	99	99	99	98.9	99.1	98.6	99	98.7	99	100	100	100	99.6	98.9	99.4	99.6	98.6	99.1
Jn-1	97.8	99.3	99.6	98.6	98.7	98.9	98.6	98.6	99.4		X	99	99.3	98.7	98.7	98.7	98.6	98.9	98.3	98.7	98.4	98.7	100	100	100	99.3	98.6	99.1	99.3	98.3	98.9
Kn-14	98.9	98.9	98.9	99.6	99.7	99.6	99.3	99.6	99	99	99	X	98.9	99.7	99.7	99.7	99.6	99.6	99	98.9	99.1	99.7	99.1	99.3	98.9	98.9	99.6	98.7	98.9	99	99.6
Kn-16	98	99.4	99.1	98.7	98.9	99	98.7	98.7	99.6	99.6	99.3	98.9	X	98.9	98.9	98.9	98.7	99	98.4	98.9	98.6	98.9	99.7	99.6	99.4	99.4	98.7	99.4	99.4	98.4	99
Gn-3	99.1	98.9	98.6	99.9	100	99.9	99.6	99.9	99	99	98.7	99.7	98.9	X	100	100	99.9	99.9	99	98.9	99.4	100	99.1	99.1	99.1	98.9	99.9	98.7	98.9	99.3	99.9
Mt-5	99.1	98.9	98.6	99.9	100	99.9	99.6	99.9	99	99	98.7	99.7	98.9	100	X	100	99.9	99.9	99	98.9	99.4	100	99.1	99.1	99.1	98.9	99.9	98.7	98.9	99.3	99.9
Mt-6	99.1	98.9	98.6	99.9	100	99.9	99.6	99.9	99	99	98.7	99.7	98.9	100	100	X	99.9	99.9	99	98.9	99.4	100	99.1	99.1	99.1	98.9	99.9	98.7	98.9	99.3	99.9
Fr-4	99	98.7	98.4	99.7	99.9	99.7	99.4	99.7	98.9	98.9	98.6	99.6	98.7	99.9	99.9	99.9	X	99.7	98.9	98.7	99.3	99.9	98.7	98.7	98.7	98.7	99.7	98.6	98.7	99.1	99.7
Na-8	99	99	98.7	99.7	99.9	100	99.4	99.7	99.1	99.1	98.9	99.6	99	99.9	99.9	99.9	99.7	X	99.1	99	99.6	99.9	99.1	99.1	99.1	99	99.7	98.9	99	99.4	99.7
Na-9	98.3	98.4	98.1	98.9	99	99.1	98.6	98.9	98.6	98.6	98.3	99	98.4	99	99	99	98.9	99.1	X	98.4	98.7	99	98.7	98.6	98.4	98.4	98.9	98.3	98.4	98.6	98.9
Mp-1	98	98.9	98.6	98.7	98.9	99	98.7	98.7	99	99	98.7	98.9	98.9	98.9	98.9	98.9	98.7	99	98.4	X	98.6	98.9	99.1	99.1	99.1	98.9	98.7	98.7	98.9	98.4	99
Ja-11	98.6	98.6	98.6	99.3	99.4	99.6	99	99.3	98.7	98.7	98.4	99.1	98.6	99.4	99.4	99.4	99.3	99.6	98.7	98.6	X	99.4	99.1	99.1	99.1	98.6	99.3	98.4	98.6	99.6	99.3
Mp-2	99.1	98.9	98.6	99.9	100	99.9	99.6	99.9	99	99	98.7	99.7	98.9	100	100	100	99.9	99.9	99	98.9	99.4	X	99.1	99.1	99.1	98.9	99.9	98.7	98.9	99.3	99.9
Ja-13	97.4	99.7	100	99.1	99.1	99.1	99	98.7	100	100	100	99.1	99.7	99.1	99.1	99.1	98.7	99.1	98.7	99.1	99.1	99.1	X	100	100	99.7	99.1	99.6	99.6	98.7	99.1
Mp-3	97.4	99.6	100	99.1	99.1	99.1	98.9	98.7	100	100	100	99.3	99.6	99.1	99.1	99.1	98.7	99.1	98.6	99.1	99.1	99.1	100	X	100	99.6	99.1	99.4	99.6	98.7	99.1
Ja-14	97.4	99.4	100	99.1	99.1	99.1	98.7	98.7	100	100	100	98.9	99.4	99.1	99.1	99.1	98.7	99.1	98.4	99.1	99.1	99.1	100	100	X	99.4	99.1	99.3	99.6	98.7	99.1
Mp-4	98	99.4	99.1	98.7	98.9	99	98.7	98.7	99.6	99.6	99.3	98.9	99.4	98.9	98.9	98.9	98.7	99	98.4	98.9	98.6	98.9	99.7	99.6	99.4	X	98.7	99.6	99.4	98.4	99
Mp-5	99	98.7	98.4	99.7	99.9	99.7	99.4	99.7	98.9	98.9	98.6	99.6	98.7	99.9	99.9	99.9	99.7	99.7	98.9	98.7	99.3	99.9	99.1	99.1	99.1	98.7	X	98.6	98.7	99.1	99.7
Ja-16	97.8	99.3	99	98.6	98.7	98.9	98.6	98.6	99.4	99.4	99.1	98.7	99.4	98.7	98.7	98.7	98.6	98.9	98.3	98.7	98.4	98.7	99.6	99.4	99.3	99.6	98.6	X	99.3	98.3	98.9
Mp-7	98	99.4	99.1	98.7	98.9	99	98.7	98.7	99.6	99.6	99.3	98.9	99.4	98.9	98.9	98.9	98.7	99	98.4	98.9	98.6	98.9	99.6	99.6	99.6	99.4	98.7	99.3	X	98.4	99
Mp-9	98.4	98.4	98.4	99.1	99.3	99.4	98.9	99.1	98.6	98.6	98.3	99	98.4	99.3	99.3	99.3	99.1	99.4	98.6	98.4	99.6	99.3	98.7	98.7	98.7	98.4	99.1	98.3	98.4	X	99.1
Sm-1	99	99	98.7	99.7	99.9	99.7	99.7	99.7	99.1	99.1	98.9	99.6	99	99.9	99.9	99.9	99.7	99.7	98.9	99	99.3	99.9	99.1	99.1	99.1	99	99.7	98.9	99	99.1	X

Table 2.10 Summary of sequence comparison of HVX CP and TGB1 genes

Nucleotide sequence % identity			
	TN	**Kr**	**US**
CP (All TN)	98.3 – 100	98.8 – 99.7	98.5 – 99.5
TGB1 (All TN)	98.1 – 100	97.4 – 99.1	X

Amino acid sequence % identity			
	TN	**Kr**	**US**
CP (All TN)	98.6 – 100	99.1 – 100	99.1 - 100
TGB1 (All TN)	98.3 – 100	97 – 98.3	X

TN = HVX Tennessee isolates

Kr = HVX Korean isolate

US = HVX US isolate

Table 2.11 Member species of *Flexiviridae* family included in phylogenetic analysis

Genus	**Virus species**	**Acronym**	**GenBank accession number**
Foveavirus	*Apple stem pitting virus*	ASPV	D21829
	Grapevine rupestris stem pitting associated virus	GRSPaV	AF026278
Vitivirus	*Grapevine virus A*	GVA	X75433
	Grapevine virus B	GVB	X75448 X75896
Trichovirus	*Apple chlorotic leaf spot virus*	ACLSV	M58152
	Potato virus T	PVT	D10172
Citrivirus	*Citrus leaf blotch virus*	CLBV	NC_003877
Allexivirus	*Shallot virus X*	ShVX	M97264
	Garlic virus A	GarV-A	AB010300.1
	Garlic virus B	GarV-B	AB010301
	Garlic virus C	GarV-C	AB010302

Table 2.11 Continued

Genus	**Virus species**	**Acronym**	**GenBank accession number**
Carlavirus	*Poplar mosaic virus*	PopMV	X65102
	Potato virus M	PVM	D14449
Potexvirus	*Potato virus X*	PVX59	AF172259
	Potato virus X	PVX41	M63141
	Potato virus X	PVX02	X55802
	Potato virus X	PVX36	AF272736
	Potato virus X	PVX18	AB056718
	Potato virus X	PVX19	AB056719
	Potato virus X	PVX76	M72416
	Potato virus X	PVX16	M95516
	Potato virus X	PVX55	NC_001455
	Potato virus X	PVX80	M38480
	Alternanthera mosaic virus	AltMV	NC_007731

Table 2.11 Continued

Genus	**Virus species**	**Acronym**	**GenBank accession number**
	Cactus virus X	CVX	NC_002815
	Papaya mosaic virus	PapMV	NC_001748
	Cassava common mosaic virus	CsCMV	U23414
	Tulip virus X	TVX	AB066288
	Plantago asiatica	PIAMV	Z21647
Undefined	*Cherry green ring mottle virus*	CGRMV	NC_001946
	Sugarcane striate mosaic associate virus	SCSMaV	NC_003870
	Banana mild mosaic virus	BanMMV	NC_002729
	Banana virus X	BanVX	AY710267
	Cherry necrotic rusty mottle virus	CNRMV	NC_002468

Appendix 2: Figures

Figure 2.1 Symptoms induced by HVX in hosta cultivars collected from different region in Tennessee. A- ‘Gold Standard’, B- ‘August Moon’, C- ‘Golden Tiara’, D- ‘So Sweet’, E- ‘So Sweet’ and F- ‘Scooter’.

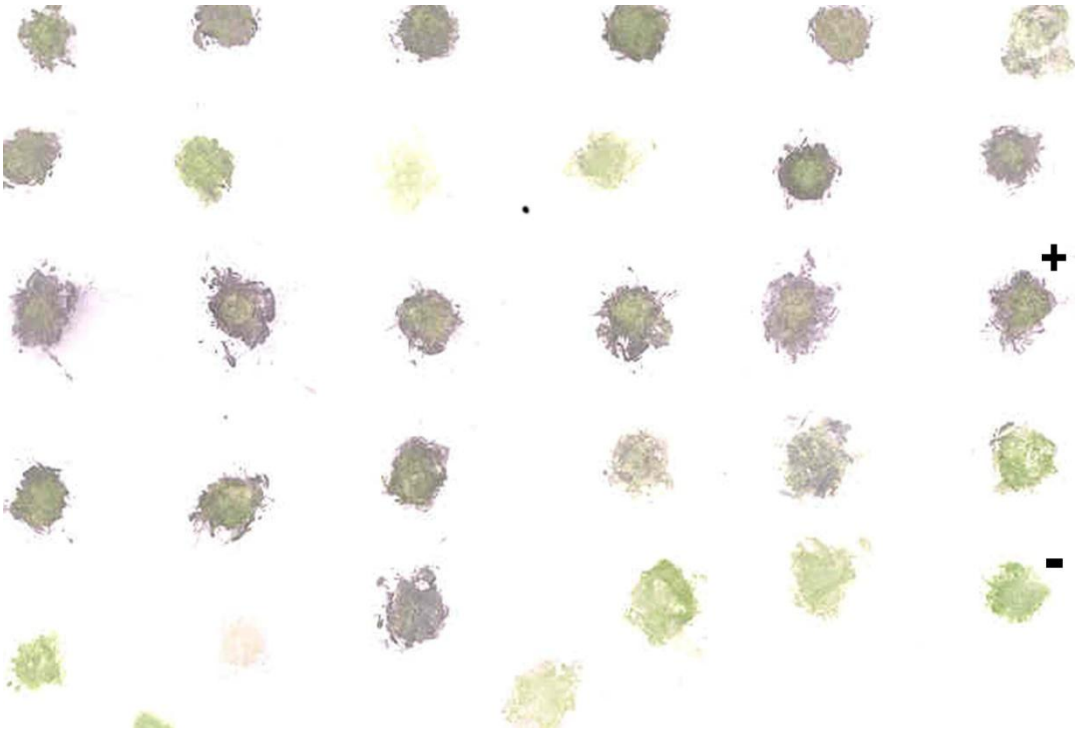

Figure 2.2 Screening of hosta leaf tissues derived from the collected hosta plants by squash immunoblotting assay for the presences HVX. An example of positive (+) or negative (-) samples are marked

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A B

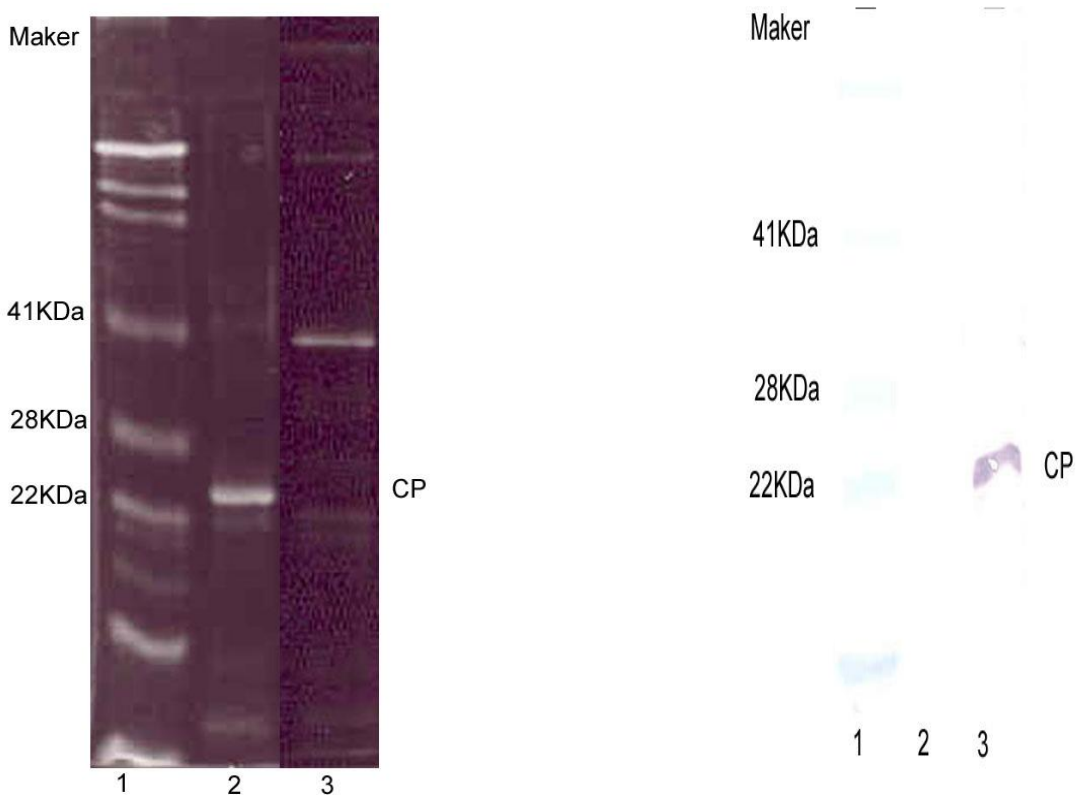

Figure 2.3 Evaluation of HVX CP molecular weight by SDS-PAGE (A) and western immunoblotting (B). Lane 3 was loaded with total protein extracted from a HVX-infected hosta cultivar 'Lady Guinevere' from Jackson. Purified AMV (Lane 2) served as a control for SDS-PAGE since its CP is about the same molecular weight as that of HVX. Lane 1 was loaded with molecular weight proteins. One gel was stained with coomassie brilliant blue (A), and a sister gel was transferred to nitrocellulose membrane and probed with polyclonal antisera against HVX CP (B). Molecular weight of HVX CP is about 23KDa.

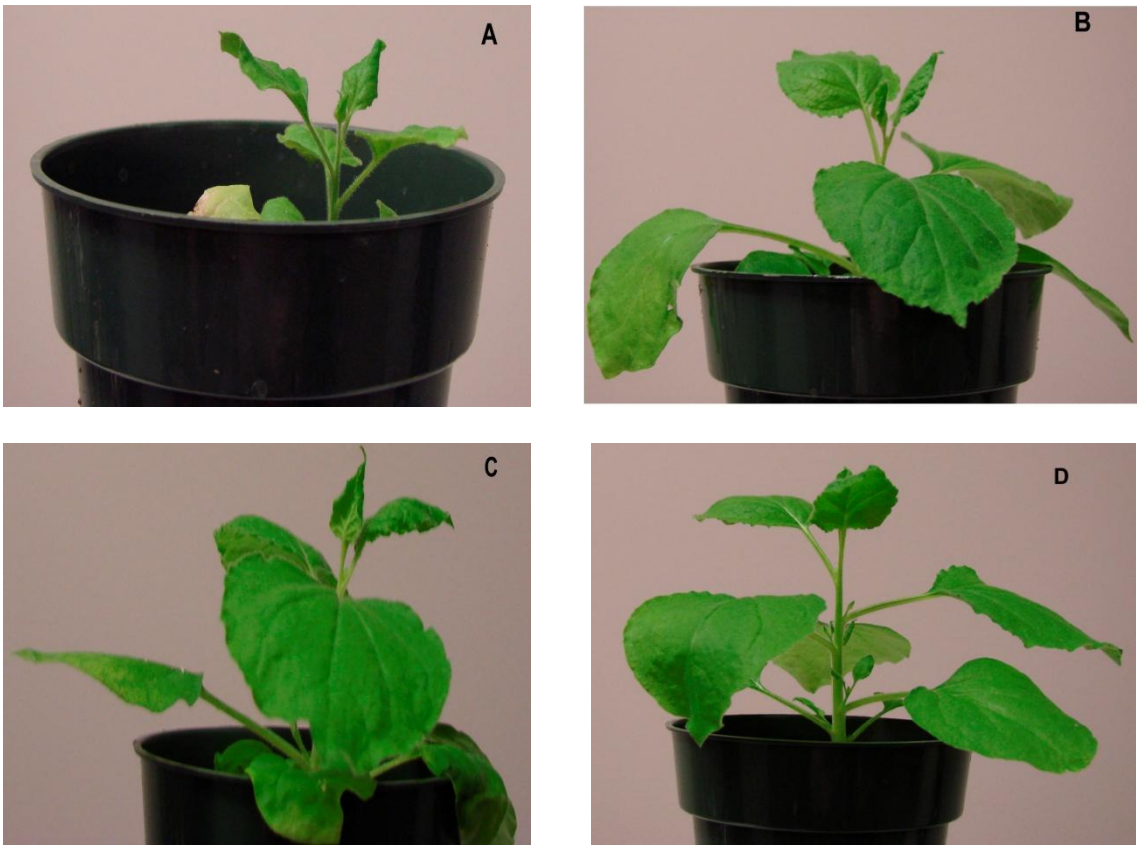

Figure 2.4 Assay of representative symptomatic hosta on *Nicotiana benthamiana* for the presence of HVX. The plants were inoculated with sap from representative HVX-infected hostas obtained from Tennessee. Plants were maintained in a growth chamber until evaluation for the presence of symptoms and photographed 14 days post-inoculation. A exhibits stunted growth, B, C and D exhibit puckering and discoloration of leaves. These plants tested positive to HVX by squash immunoblotting assay.

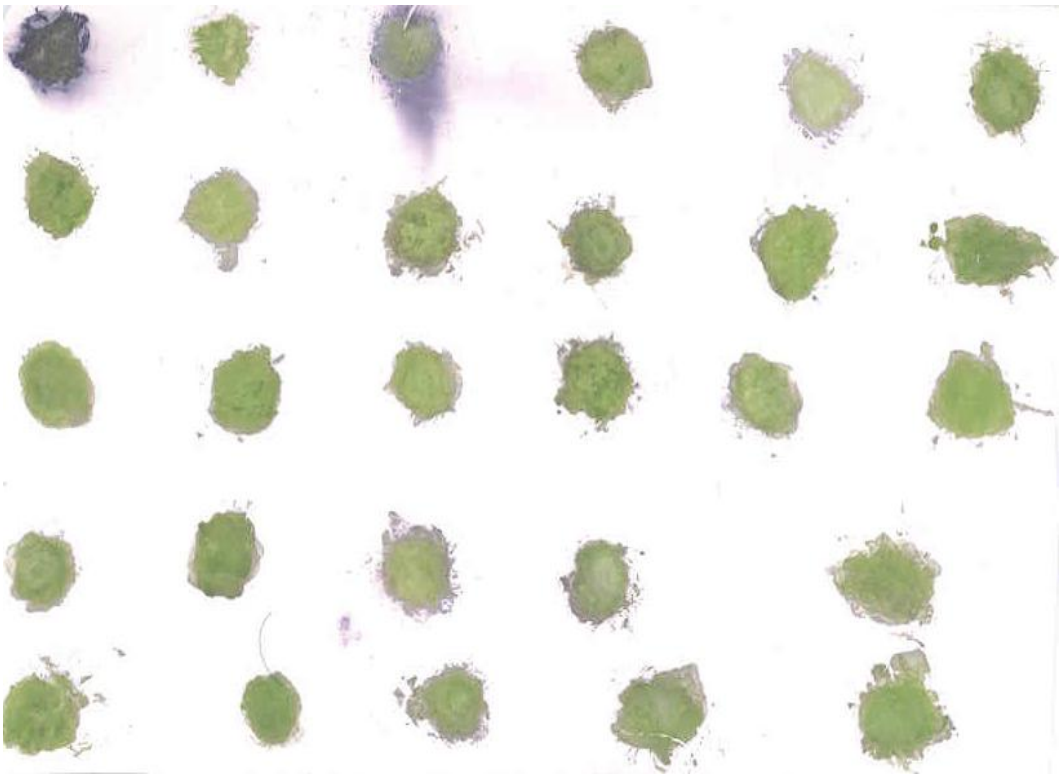

Figure 2.5 Squash immunoblotting assay for detection of HVX in mechanically inoculated *Nicotiana benthamiana* 14 days post-inoculation with sap from HVX infected hostas. Representative HVX isolates from the three regions of Tennessee were assayed.

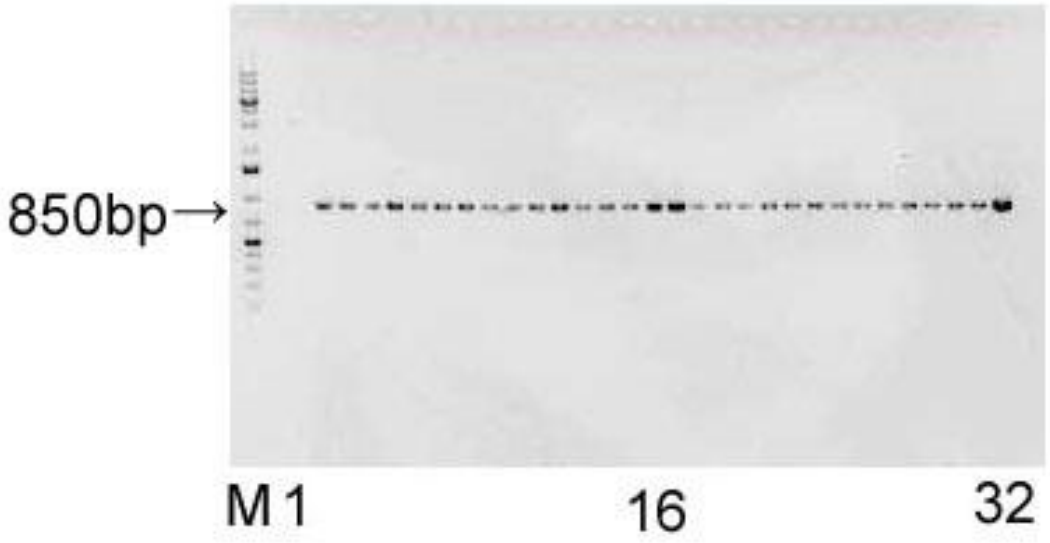

Figure 2.6 Reverse-transcription polymerase chain reaction (RT-PCR) amplification of HVX CP gene generated from thirty HVX-infected hostas obtained from Tennessee (Lanes3-32). RT-PCR was performed with primers HVXs5639 and HVXa6488 and produced amplicons of 850bp. Lane 1 was loaded with PCR products generated in the absence of any template. RNA generated from HVX-free hosta was used in lane 2 as a negative control. Molecular markers were loaded in lane M.

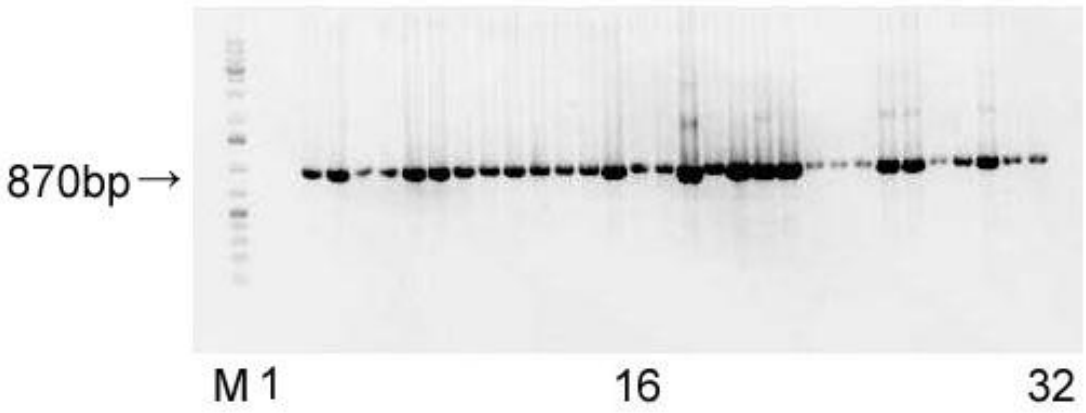

Figure 2.7 RT-PCR products of TGB1 gene from HVX-infected hostas collected from different regions of Tennessee (lane 3-32). Molecular markers (1kb DNA Ladder) were loaded in lane M. Lane 1 was loaded with PCR products generated in the absence of any template. Lane 2 was loaded with RT-PCR product generated from HVX-free hosta and served as a negative control. PCR was performed with primers HVXs4501 and HVXa5377, and produced amplicons of 870bp.

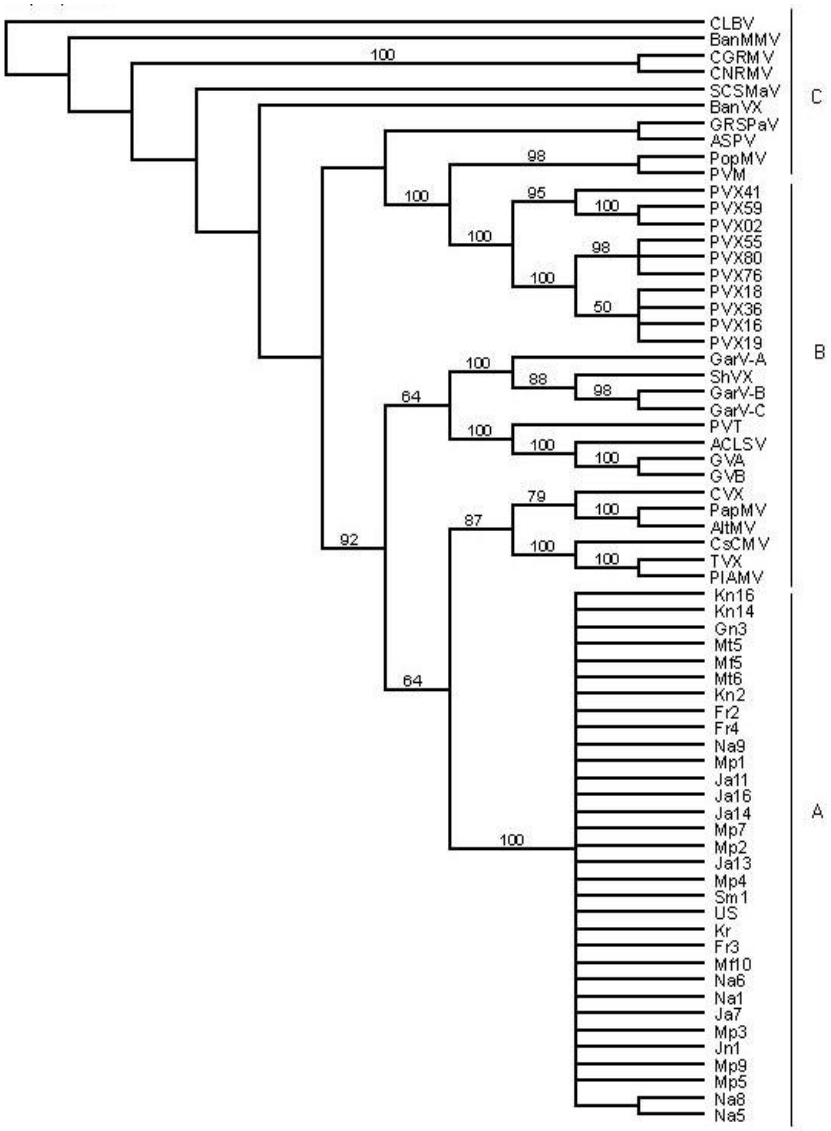

Figure 2.8 Phylogenetic tree inferred from parsimony analysis of amino acid sequences of CP. Numbers above each branch are the neighbor-joining bootstrap scores given as percentage of 1000 replicates. Group A include all HVX isolates, group B contains potexviruses while group C contains representative species from other genera in the *Flexiviridae* family. Besides HVX TN isolates, other sequences were obtained from the GenBank database. Accession numbers and full names the viruses are listed in table 2.11.

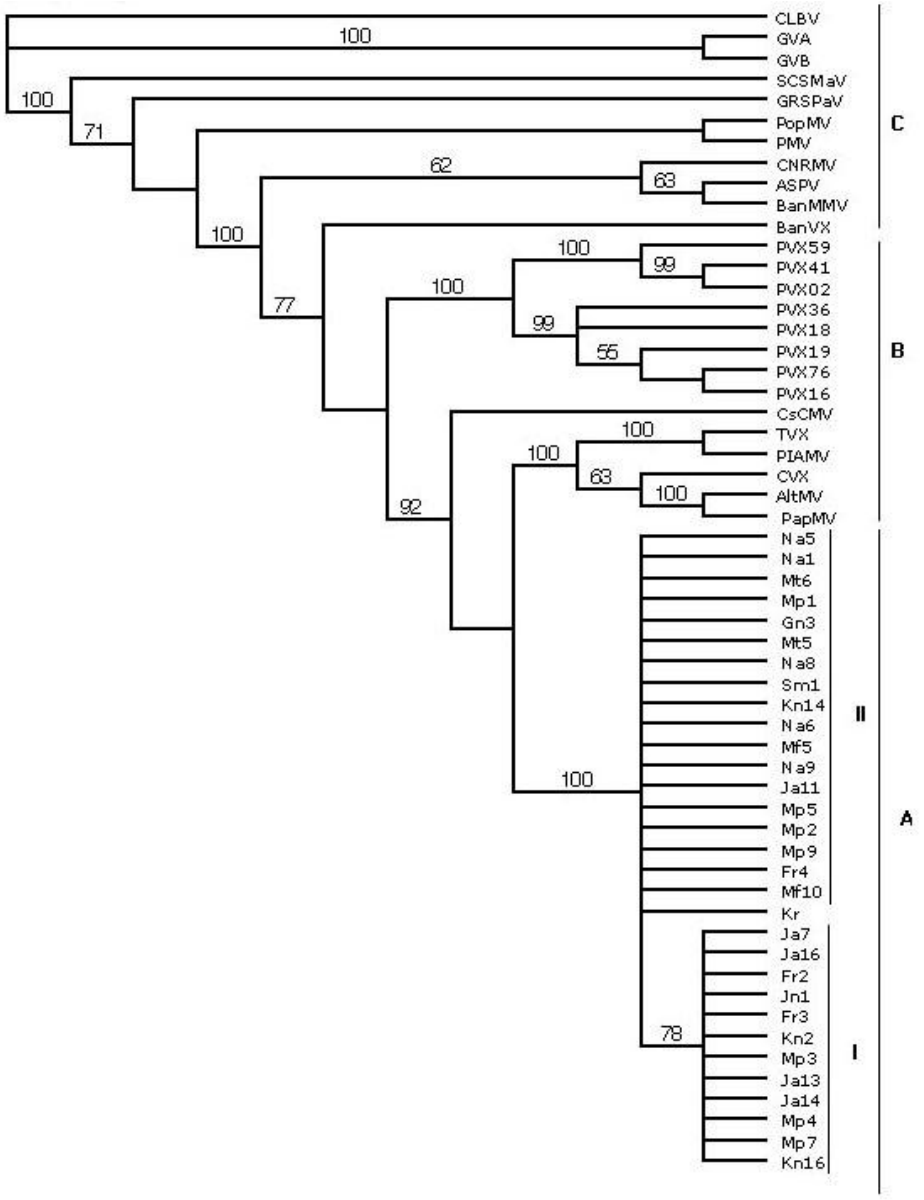

Figure 2.9 Phylogenetic tree inferred from parsimony analysis of amino acid sequences of TGB1. Numbers above each branch are the neighbor-joining bootstrap scores given as percentage of 1000 replicates. Group A include all HVX isolates including HVX Kr isolate, group B contains potexviruses while group C contains representative species from other genera in the *Flexiviridae* family. Besides HVX TN isolates, other sequences were obtained from the GenBank database. Accession numbers and full names of the viruses are listed in table 2.11

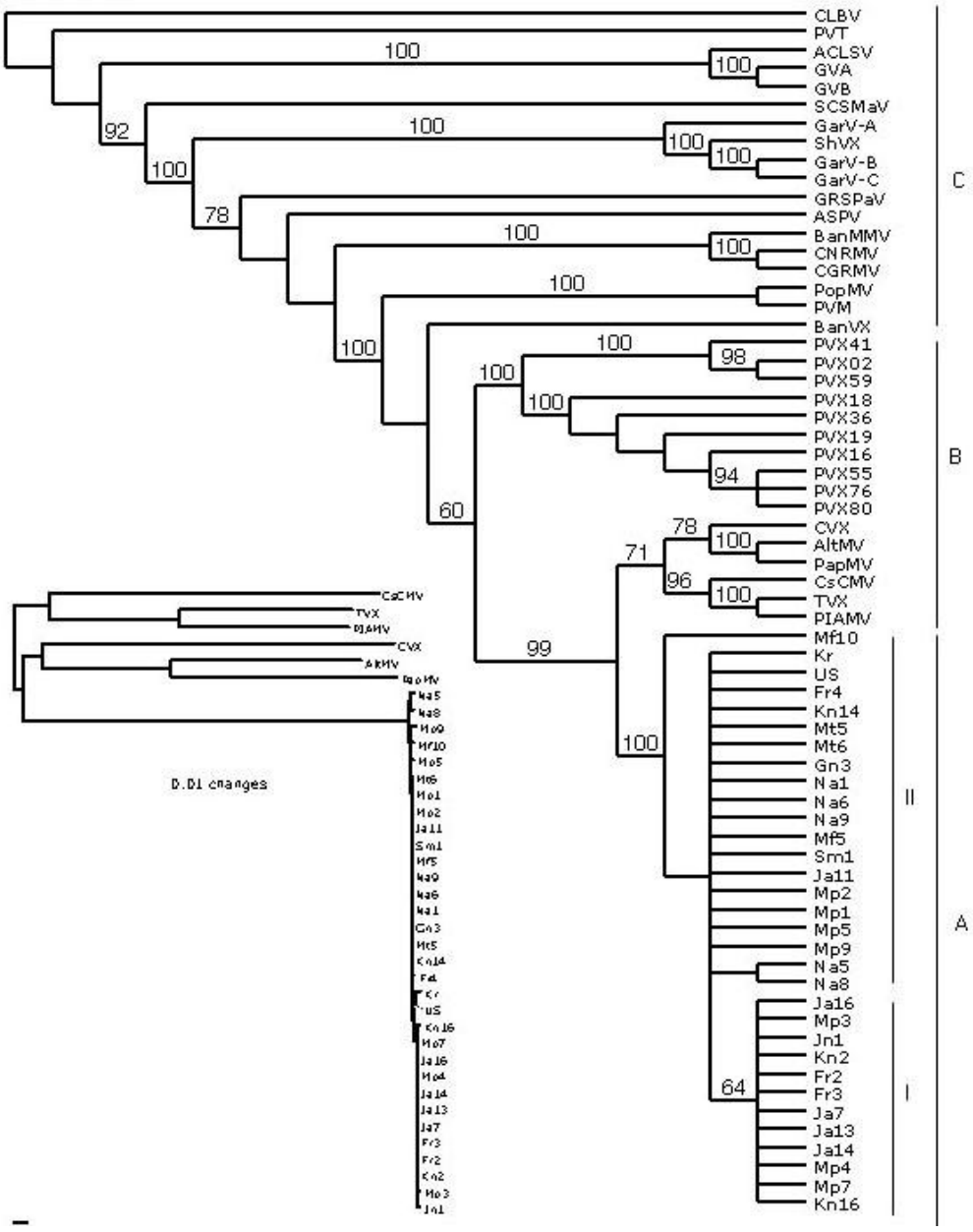

Figure 2.10 Phylogenetic tree inferred from parsimony analysis of amino acid sequences of TGB1 and CP combined. Numbers above each branch are the neighbor-joining bootstrap scores given as percentage of 1000 replicates. Group A include all HVX isolates, group B contains potexviruses while group C contains representative species from other genera in the *Flexiviridae* family. Besides HVX TN isolates, other sequences were obtained from the GenBank database. Accession numbers and full names of the viruses are listed in table 2.11.

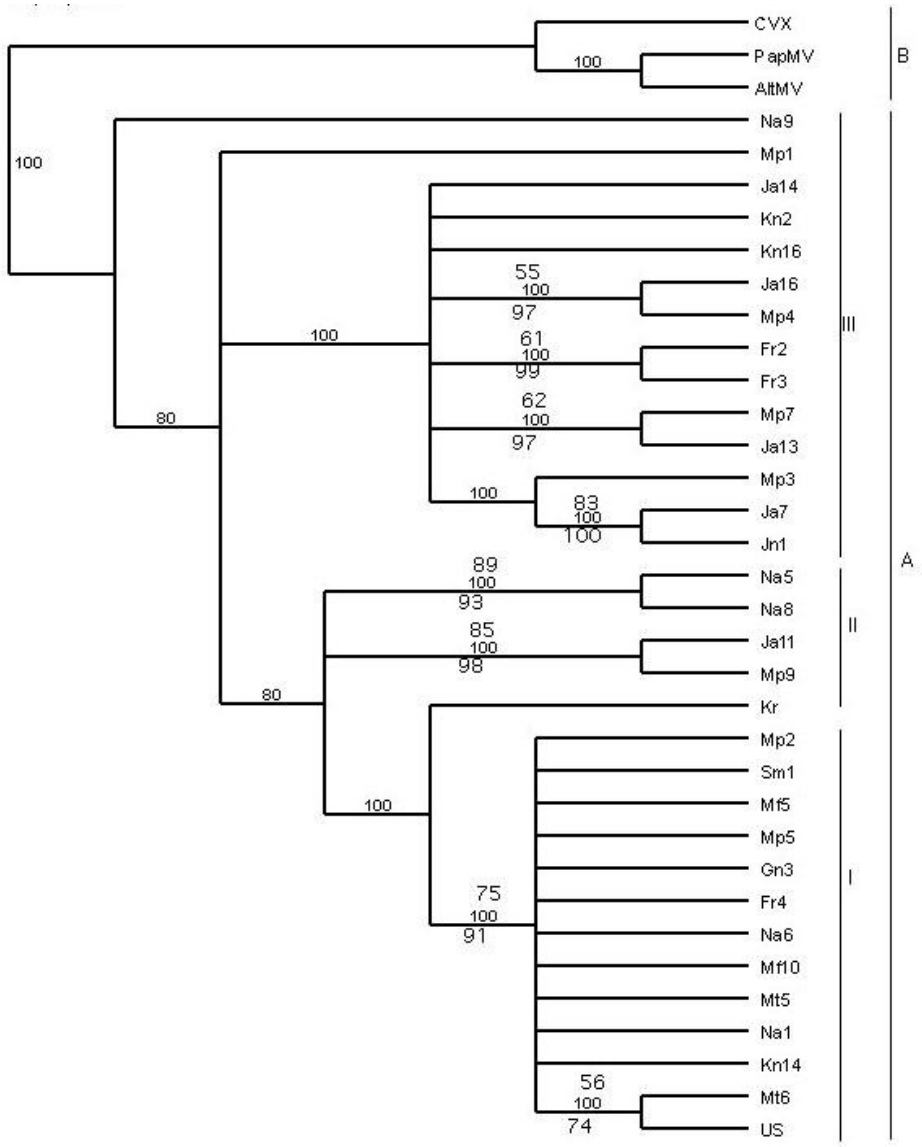

Figure 2.11 Phylogenetic tree inferred from parsimony analysis of nucleotide sequences of TGB1 and CP combined. Two numbers above and below branch are the parsimony bootstrap and bayesian scores, respectively. Bootstrap scores are indicated as percentage of 1000 replicates. Group A include all HVX isolates, group B contains potexviruses. Sequences of the potexviruses and HVX Kr- and US-isolates, were obtained from the GenBank database. Accession numbers and full names of the viruses are listed in table 2.11.

CHAPTER III

Development of a Sensitive Assay for *Hosta virus X* in Large Scale Screening

Abstract

A simple, rapid, reliable and sensitive procedure based on reverse transcriptase–polymerase chain reaction (RT-PCR) combined with restriction fragment length polymorphism assay (RFLP) was developed for the detection of *Hosta virus X* (HVX) in hosta. This method was based on knowledge of high genetic homogeneity of HVX coat protein (CP) gene. CP nucleotide sequence analyses of thirty selected HVX Tennessee (TN) isolates and available HVX CP sequences in GenBank revealed high level of sequence similarity among all the isolates. Sets of primers were designed from the conserved regions of the CP gene. Restriction sites common among all HVX isolates were determined. CP-specific primers, HVXs55 and HVXa598, generated amplicons of 544 base pairs. Digestion of amplicons with *Hind* II endonuclease resulted into two distinct fragments following electrophoresis. This RT-PCR-RFLP method was successfully applied to all HVX TN isolates and showed to be specific and sensitive in detecting HVX in hostas. RT-PCR followed by restriction digest analysis easily detected HVX in composite sample containing one leaf disc (approximately 0.1cm in diameter) of HVX-infected hosta and up to 200 discs of HVX-free hosta leaves. When a different HVX-infected hosta cultivar served as the source of the infected disc, similar results were obtained. Thus, RT-PCR followed by RFLP analysis is a highly sensitive and reliable method for screening hostas for the presence of HVX on a large scale.

3.1 Introduction

In the USA, several viruses have been reported to infect hostas. HVX is the most economically important plant virus found in hosta (6). HVX is a member of the genus *Potexvirus* within the family *Flexiviridae*. HVX is a positive-sense ssRNA of 6.4Kb in size and possesses five open reading frames (ORFs) which are translated into at least three functional proteins. The first and largest ORF is the polymerase gene. Viral movement proteins (MPs) are translated from the three central ORFs known as triple gene block (TGB) 1, 2 and 3. Besides function as a movement protein, TGB1 possesses RNA silencing suppressor and helicase activities. The viral coat protein (CP) gene located downstream of the triple gene block is translated from ORF 5 via a subgenomic RNA (12, 16).

HVX has a narrow host range and naturally infects only hosta. The virus is commonly transmitted through any mechanical means that involve the transfer of sap from infected plant to a susceptible one (7). Symptoms induced by HVX vary and include mosaic, necrosis, leave deformation and reduced plant growth (7, 14). HVX is regarded as the main pathogen of hosta and has been a matter of concern to nurseries, garden centers and home gardeners. Since HVX infection is latent in some hosta cultivars and induces no symptoms, this presents challenges for hosta growers as these symptomless cultivars serve as a source of inoculum. Moreover, HVX antigen reacted to clover yellow mosaic virus (ClYMV) antiserum and hydrangea ringspot virus (HyRSV) antiserum (3). Hence, accurate diagnosis of HVX becomes an integral part of any effective management of the virus in hosta.

Serological assays have served as the major diagnostic tests for screening of HVX in hostas. These include enzyme-linked immunosorbent assay (ELISA) (7), squash immunoblotting assay (1) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (3). Biological assays revealed that *Nicotiana benthamiana* is the only known indicator plant for HVX screening (3). Particles of HVX are also an important means of virus identification using an electron microscope or immunosorbent electron microscope (3). In addition, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) that is a powerful tool has been used in HVX diagnostics (1, 12).

RT-PCR has been developed for the diagnosis of several viruses and has been shown to be more specific with many other advantages over serological and biological assays (17). Serological assays can give a false positive result (10). Moreover, cross-reactivity can occur between antigen and antiserum from different viruses. An example is the reaction of HVX antigen with antiserum to ClYMV and vise-versa (3). Bioassay is laborious, time-consuming and requires various species of indicator plants (11), however, this is not much of application to HVX in hosta due to very narrow host range of the virus (3). RT-PCR followed by restriction digestion and electrophoresis analysis has great potential to simplify virus identification and strain differentiation.

Restriction fragment length polymorphism (RFLP) is a useful tool for confirmation and verification of PCR products. Xu and Nie (17) used RFLP following RT-PCR for identification and confirmation of *Alfalfa mosaic virus* (AMV) in potatoes. Similarly, Xu *et al* (18) verified the presence of *Potato mop-top virus* in potatoes using RT-PCR-RFLP. RFLP is also an important assay for classifying virus isolates into distinct groups as demonstrated by Parrella *et al* (13). They utilized RT-PCR followed by

RFLP to identify and classify AMV isolates into two subgroups. Marie-Jeanne *et a*l (9) used RT-PCR and restriction enzyme digest analyses to differentiate potyviruses infecting *Poaceae*. Furthermore, RFLP is a useful technique for studying genetic variability of virus isolates. Fattouch *et al* (4) studied the genetic diversity of *Grapevine fanleaf virus* (GFLV) using RFLP analysis and showed that GFLV in Tunisia consists of two restrictotypes. RFLP analysis was used to differentiate *Cherry leaf roll virus* isolates belonging to different phylogenetic groups (2).

In order to develop RT-PCR-RFLP technique for specific and sensitive detection of HVX in hosta, sets of primers were designed from the conserved regions of the CP derived from thirty HVX Tennessee (TN) isolates as well as those available in GenBank. A restriction endonuclease with the site conserved among all HVX isolates was used to carry out RFLP analysis. This confirmed the identity of PCR amplicons. This chapter describes the development of RT-PC-RFLP as a useful technique for HVX detection in hosta. Screening for HVX in large scale hosta production by this technique is also demonstrated. Moreover, this chapter also presents responses of hosta cultivars to HVX in comparison to previous published reports on responses of hosta cultivars to inoculation with the virus.

3.2 Materials and methods

A. Hosta samples

Hostas with virus-like symptoms were collected from different geographical regions of Tennessee. These were obtained from nurseries, local growers, gardens, and retail outlets. A total of 82 plants constituting 31 different cultivars were collected (Table

2.2). The infected hostas were maintained in a light–temperature–controlled growth chamber (Environmental Growth Chamber, USA) at 25^{o}C with 16 hours light: 8 hours dark photoperiod cycle and 46% humidity. All the plants were indexed for the presence of HVX by squash immunoblotting assay. Table 3.1 shows reaction of hosta cultivars to HVX infection as found in this work and compared to previous studies.

B. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from hostas, verified to be infected with HVX by squash immunoblotting assay. Total RNA was extracted from thirty different serologically HVX–positive hostas using RNeasy Plant Mini Kit (Qiagen, USA) following the manufacturer's instructions. RNA extracted from leaves of HVX-free hosta was used as a negative control in all subsequent experiments. All tubes and buffer used were ribonuclease free. Total RNA was quantified using a smart spectrophotometer (Bio-Rad, USA). RNA extracts were used immediately or stored at -20^{o}C until required.

First-strand cDNA synthesis was carried out using Super Script III Reverse Transcriptase (SS III RT) (Invitrogen, USA). A mixture of 1µl 10pmol oligo (dT) primer (Integrated DNA, USA), 10µl total RNA extract, 1µl of 2.5mM dNTPs (Takara, Japan) and 2.5µl distilled water was heated at 65^{o}C for 5 min. After incubation in ice for 2 min, 0.5µl of SS III RT, 4µl of 5X first strand buffer and 1µl of 0.1M dithiothreitol (Invitrogen) were added to the mixture. The 20µl reaction was heated at 50^{o}C for 45 min and then at 70^{o}C for 15 min in a Peltier Thermal Cycle (MJ Research, USA).

A pair of primers was designed in this study for the amplification of HVX CP based on the sequences of HVX obtained from the NCBI website (Table 3.2).

Amplification of the entire CP gene was performed with Ex *Taq* polymerase (Takara) using 10X reaction buffer provided in a Peltier Thermal Cycler (MJ Research). Reaction mixture of 50µl consisted of 2µl forward primer HVXs5639 (Table 3.2), 2µl reverse primers HVX6488 (Table 3.2), 2µl of dNTPs, 0.25µl of Ex *Taq*, 5µl of 10X reaction buffer and 36.75µl of distilled water. The amplification cycle was as follows: initial denaturation for 4 min at 94^{o}C, followed by 35 cycles of 95^{o}C for 30 sec, 56^{o}C for 30 sec and 72^{o}C for 30 sec. The final extension was at 72^{o}C for 10 min. A reaction containing cDNA generated from HVX-free hosta and another reaction with no cDNA were included in all the PCRs as negative controls. PCR amplified CP fragments were analyzed by electrophoresis in 1% agarose gel (FisherBiotech, USA) in the presence of ethidium bromide (FisherBiotech) and visualized under UV universal hood (Bio-Rad, USA). PCR products were purified using MinElute PCR Purification Kit (Qiagen) following the manufacturer's instructions.

C. Nucleotide sequence determination and analysis

Two pairs of primers, which included the reverse and forward primers used for PCR amplification and internal primers, were used for the completion of full length CP sequences (Table 3.2). Purified PCR products were quantified and submitted to the sequencing laboratory, Molecular Biology facility, University of Tennessee, to be sequenced bi-directionally using an ABI 3730 DNA analyzer (Applied Biosystems). Sequences obtained were edited and assembled with vector NTI software (Invitrogen). CP Nucleotide sequences of the 30 HVX TN isolates were obtained (Table 2.5). Multiple sequence alignments of the CP sequences and HVX CP sequences obtained from the

GenBank were generated with clusterW2 (EBI website). Sets of primers were designed from the regions conserved among these isolates using Vector NTI software (Table 3.3). In addition, the unique endonuclease restriction sites common among the isolates were determined using Vector NTI (Table 3.4).

D. Restriction enzyme digestion

Restriction endonuclease *Hind* II (Takara) was used to digest all the PCR products. Based on the analyses of sequences of all the HVX isolates examined, the amplified CP region contains one *Hind* II site hence; the PCR product digested should generate only two fragments. Restriction digestion was carried out in a total volume of 20µl using 5µl of the amplified PCR product, 0.5µl of restriction enzyme, 2µl of 10X M buffer and 12.5µl of distilled water. The enzymatic restriction was performed at 37^{o}C for 1 hr. The digested DNAs were subjected to electrophoresis using 2% agarose gel (FisherBiotech) in the presence of ethidium bromide. A mixture of 6X MassRuler DNA loading dye (Fermentas, Canada) and the digested product (1:5) was loaded into each well, and 2µl of 1Kb O' GeneRulers DNA ladder (Fermentas) was also subjected to electrophoresis in a parallel lane. Electrophoresis was done at 100 voltage until the tracking dye (bromophenol blue) had migrated 3/4 of the length of the gel towards the positive electrode. The gel was then visualized and photographed under a UV universal hood (Bio-Rad).

E. Evaluation of RT-PCR-RFLP and detection of HVX in large scale sampling

To evaluate the efficiency of the RT-PCR-RFLP method, primers HVXs55 and HVXa598 (Table 3.3) generated from the conserved regions of CP were utilized to amplify partial region of all the 30 HVX TN isolates. Amplicons of CP (544bp) were digested with *Hind* II. The digestion consistently yielded two distinct fragments for all of the isolates examined.

Moreover, the efficiency of the RT-PCR-RFLP in detecting HVX in a large scale sampling was assessed. A single leaf disc (~0.1 cm in diameter) from an HVX-infected hosta was mixed with similar sized leaf discs from HVX-free hostas at ratios of 1:0, 1:5, 1:10, 1:20, 1:40, 1:80 and 1:100 (HVX-infected to HVX-free discs), respectively. Hosta cultivar 'Yellow Splash' infected with HVX isolate Na-5 containing two unique amino acids in CP and distinct from other isolates served as a source of HVX-infected tissues. Each sample was pulverized in liquid nitrogen. Total RNA was extracted from each sample only from 0.1g of pulverized leave tissues. RNA extracts were quantified and the same concentration (750µg) was used to generate the first-strand cDNA. RT-PCR was carried out as mentioned above with primers HVXs55 and HVXa598 (Table 3.3). All PCR products were digested with *Hind* II restriction enzyme. Purified PCR products generated from infected hosta, and from mixture of infected and HVX-free hosta leaf discs at ratio 1:80 and 1:100 were sequenced. The nucleotide sequences and the deduced amino acid sequences were compared using vector NTI program. The experiment was repeated using a different HVX-infected hosta cultivar 'So Sweet' as the source of infected tissues.

3.3 Results

A. Reaction of hosta cultivars to HVX

From the eighty-two hosta plants sampled, sixty-one tested positive to HVX by squash immunoblotting assay (Table 2.2). These consisted of different hosta cultivars. The presence of HVX in some of these cultivars had not been reported earlier in literature. These included cultivars 'Karin', 'Lady Guinevere', 'Scooter', 'Spritzer' 'Blue Diamond' and 'Austin Dickson' (Table 3.1). More interestingly, hosta cultivar 'Frances Williams' that previously was reported to be resistant to HVX (8) was found to be susceptible to the virus (Table 3.1). The susceptibility of this cultivar was confirmed by squash immunoblotting and RT-PCR analyses.

B. RT-PCR

The CP genes of thirty selected HVX-isolates were amplified by RT-PCR. Each of these isolate amplified by RT-PCR yielded an amplicon of 850bp (Figure 2.6). There was no PCR product in the control reaction. Three representative PCR amplicons digested by *Hind* II yielded two fragments of expected sizes of 458 and 392 bp (Figure 3.1)

C. Sequence analysis

Nucleotide sequences of the CP gene of HVX TN isolates were generated and their comparison with those present in GenBank showed above 98% sequence identity (Table 2.9). Multiple sequence alignments of CP nucleotide sequences, including 30 TN isolates obtained in this study and those previously reported revealed common conserved

regions scattered across CP sequences. Sets of primers were designed based on these conserved regions. Table 3.3 shows the different primers, their positions within CP and their sequences. Moreover, analysis of the nucleotide sequences using Vector NTI showed unique endonuclease restriction sites common among these isolates. Table 3.4 contains list of restriction enzymes and their sites.

D. Molecular diagnosis of HVX by RT-PCR-RFLP

Primers HVXs55 and HVXa598 designed from CP conserved regions generated PCR amplicon of 544bp from all HVX TN isolates examined (Figure 3.2). The primers were specific for all HVX isolates and did not produce any amplicon from RNA extracts derived from a HVX-free hosta. All PCR amplicons were digested with *Hind* II into two distinct fragments; 219bp and 324bp (Figure 3.3). RFLP analysis served as a confirmatory test for RT-PCR detection method.

To evaluate the sensitivity of RT-PCR-RFLP for the detection of HVX in hosta, a leaf disc from an HVX-infected hosta was mixed with discs derived from a HVX-free hosta plant in a ratio from 1:0 to 1:100. A reliable PCR amplicons of 544bp was generated from all composite samples and 2 distinct fragments were obtained following digestion with *Hind* II (Figure 3.4). Comparison of CP nucleotide and amino acid sequences generated from the original HVX-infected hosta and those generated from mixture of infected and HVX-free leaves at the ratio 1:80 and 1:100 gave 100% identity. Since original HVX isolate used in this experiment possesses amino acid sequences distinct from all the other isolates used in this study, sequences of amplified amplicon verified the authenticity of PCR amplicon generated from the composite samples.

Moreover, this experiment was reproducible with different hosta cultivars as source of infected leaf tissues (Figures 3.5).

3.4 Discussion

High infection rate of HVX in hostas calls for increased management of the virus by growers and retailers. The availability of a reliable and economically feasible diagnostic assay is essential for successful screening of hostas for the presence of HVX and the elimination of the infected plants at very early stage. CP nucleotide sequence analysis of thirty selected TN isolates with those previously reported showed that all HVX isolates are closely related and have higher than 98% sequence identity. Based on the high level of sequence conservation among the isolates, RT-PCR-RFLP was developed to facilitate HVX detection. CP-specific primers, HVXs55 and HVXa598, designed from conserved CP regions successfully amplified CP sequences from all the HVX-infected hostas obtained from different regions of Tennessee. These primers were specific for HVX and did not produce amplicons with RNA extracted from HVX-free hosta. More than five sets of HVX CP-specific primers were designed from conserved CP regions (Table 3.3), however, primers HVXs55 and HVXa598 were preferentially used to amplify all HVX TN isolates. These primers generated the largest size of PCR amplicons, and upon enzymatic digestion resulted in two distinct fragments following electrophoresis.

Furthermore, digestion of all PCR amplicons with *Hind* II verified that amplicons were derived from HVX sequences. A single *Hind* II site is conserved among all the HVX isolates examined and located in a region flanked by HVXs55 and HVXa598

primers, and other CP-specific primers designed in this study. RFLP analysis served as a confirmatory test for PCR amplicons and will reveal any false-positive result that may arise from RT-PCR amplifications. The *Hind* II was preferentially selected from all restriction endonuclease with site conserved among all HVX isolates. *Hind* II is a cheap enzyme as compared to other candidates such as *Afe*I and *Ava*II restriction enzymes. It is commonly used and readily available from many commercial sources. *Hind* II will generate two distinct bands when used to digest amplicons generated using most of the CP-specific primers listed in Table 3.3. Of all HVX isolates, MP-2 (Table 2.4) is the only isolate without a *Bgl* II restriction site; this might have resulted from mutation at the restriction site. RFLP analysis will be useful in future, when more HVX sequences would be known. It may be also useful for grouping of HVX isolates or strain differentiation.

RT-PCR combined with RFLP was not only effective in detecting HVX in individual hosta samples, but also was an efficient method of detecting HVX CP in composite samples and can be used on large scale screening of hostas. The approach of using composite samples will greatly reduce the cost and time associated with screening of hostas on a large scale for the presence or absence of HVX. Amino acids unique to HVX isolate used as a source of infection was reproduced from sequencing of PCR product of 1:80 and 1:100 composite samples. This provided evidence that PCR amplicon was specific and originated from the infected source and not a contaminant. Ability to reproduce the above results using a different HVX-infected hosta cultivar as a source for infected tissues showed that RT-PCR-RFLP analysis of composite samples is cultivar independent and seems reliable. This technique is highly sensitive for detection of HVX irrespective of the virus concentration in the infected leaf tissues.

In conclusion, RT-PCR combined with RFLP provides a rapid, sensitive and reliable detection method for HVX in hosta. This method is both cost and time efficient for screening HVX on large scale hosta production compared to commercially available HVX screening kit currently in use that allows for individual hosta testing at a time. Thus this method could provide an effective management strategy for HVX control in hostas starting from the growers and large scale producers. In addition, RFLP analysis is a promising tool for characterizing sequence variation among HVX isolates.

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Appendix 3: Tables

Table 3.1 Reaction of hosta cultivars to HVX infection

#	**Cultivar**	**HVX susceptibility**	**Reference**	**This work- HVX screening**			**Remark**
				Squash blot	**Agdia strip**	**RT-PCR-RFLP**	
1	Blue Cadet	+	2, 6	+	*	*	Confirm previous report
2	Bressingham Blue	+	5	+	*	+	Confirm previous report
3	Frances willliams	-	8	+	*	*	Now susceptible
4	Karin	No previous report	-	+	*	*	New report
5	Hardspen Lavender	No previous report	-	-	*	*	New report
6	Minuteman	No previous report	-	-	*	*	New report
7	Sum and Substance	+	5, 15	+	+	+	Confirm previous report
8	Christmas Tree	+	8	-	*	*	Needs confirmation
9	Sagae	+	15	-	*	*	Needs confirmation

Table 3.1 Continued

#	**Cultivar**	**HVX susceptibility**	**Reference**	**This work- HVX screening**			**Remark**
				Squash blot	**Agdia strip**	**RT-PCR-RFLP**	
10	Lady Guinevere	No previous report	-	+	*	+	New Report
11	Gold Standard	+	8, 15	+	*	+	Confirm previous report
12	Fragrant Blue	+	8	-	-	*	Needs confirmation
13	Albo Marginata	+	3, 5	+	+	+	Confirm previous report
14	Aureo Marginata	+	5, 8	+	+	+	Confirm previous report
15	Medio Variegata	+	5	+	+	+	Confirm previous report
16	Yellow Splash	+	3, 7, 15	+	+	+	Confirm previous report
17	Antioch	+	8	+	+	+	Confirm previous report
18	Royal standard	+	3, 7, 15	+	+	+	Confirm previous report
19	Lancifolia	+	6, 7	+	+	+	Confirm previous report

Table 3.1 Continued

#	**Cultivar**	**HVX susceptibility**	**Reference**	**This work- HVX screening**			**Remark**
				Squash blot	**Agdia strip**	**RT-PCR-RFLP**	
20	Revolution	+	15	+	+	+	Confirm previous report
21	So Sweet	+	8, 15	+	+	+	Confirm previous report
22	Wide Brim	+	5, 8	+	+	+	Confirm previous report
23	Scooter	No previous report	-	+	+	*	New report
24	Spritzer	No previous report	-	+	+	+	New report
25	Blue Diamond	No previous report	-	+	+	+	New report
26	Golden Tiara	+	7, 8, 15	+	*	+	Confirm previous report
27	August Moon	+	5, 8	+	+	+	Confirm previous report
28	Pilgrim	+	5	+	+	+	Confirm previous report
29	Moorheim	No previous report	-	-	*	*	New report

Table 3.1 Continued

#	**Cultivar**	**HVX susceptibility**	**Reference**	**This work- HVX screening**			**Remark**
				Squash blot	**Agdia strip**	**RT-PCR-RFLP**	
30	Gold Standard	+	15	+	+	+	Confirm previous report
31	Austin Dickinson	No previous report	-	+	*	+	New report

Symbols indicates positive test (+) or negative test (-) results.* indicate test was not carried out

Table 3.2 Primers used for RT-PCR amplification and sequencing of HVX CP

Primer Name	Sequence 5' – 3'	Position[1] on HVX genome	Usage
HVXs – 5639	TTCTAGAAGGAAACTGCGCT	5639 – 5658	PCR & Sequencing
HVXs 6014 (internal primer)	GCCAGGCACAAC GGTCAACTAC	6014 – 6035	Sequencing
HVXa 6169 (internal primer)	TCTTTGTAGCCCATCGCCGC	6169 – 6149	Sequencing
HVXa6488	TTAGTGGAACGGTTAGCC C	6488 – 6470	PCR & Sequencing
Oligo dt	*TTT TTT TTT TTT TTT*[2]	-	First strand cDNA synthesis

[1]Sequence position is based on the complete HVX sequence (GenBank accession number AJ620114).

[2] Non viral sequence are italicized

Table 3.3 Sequences of primers designed from conserved regions of CP gene of HVX isolates

	Primer name	**Sequence 5' – 3'**	**GC content**	**Tm**	**Length**
A	HVXs55	GCACCAACACAGGAACAACTG	52	62	21
B	HVXs112	CCAAGCCCCGATGTGCTCAAC	61	58	21
C	HVXs181	TCGTCCACAGCCATAGCGCTG	61	58	21
D	HVXs211	TGCTACCACTCAGGGTCCTC	60	56	20
E	HVXs257	GCCAGGCACAACGGTCAACTAC	61	57	22
F	HVXa598	TTGTCACCCTGCCGTCAGTGG	61	58	21
G	HVXa516	AGGAGCTCCTCCTCTGTTGG	60	56	20
H	HVXa491	ATTAGGCCTCCCGTGGGTTG	60	57	20
I	HVXa413	TCTTTGTAGCCCATCGCCGC	60	59.6	20
J	HVXa394	CCAGTTGGCTGGTGGAATC	57	54	19

Table 3.4 Restriction enzymes with unique sites conserved among CP genes of all HVX isolates examined

Enzyme name	**Restriction sequence**	***Restriction site on CP**	**Predicted fragments sizes after digest (bp)**
Aat I	AGCCT	486	112 & 431
Acp II	CCANNNNNTGG	351	247 & 296
Afe I	AGCGCT	198	143 & 400
Ava II	GGNCC	225	170 & 373
Bfa I	CTAG	308	253 & 290
Bgl I	GCCNNNNNGGC	392	206 & 337
**Bg*l II	AGATCT	135	80 & 463
Hind II	GTYRAC	274	219 & 324

Amplicons generated with primers HVXs55 and HVXa598PCR

**Bgl* II restriction is absent in Mp-2 isolate

* Site on CP are identified starting from the start codon

Appendix 3: Figures

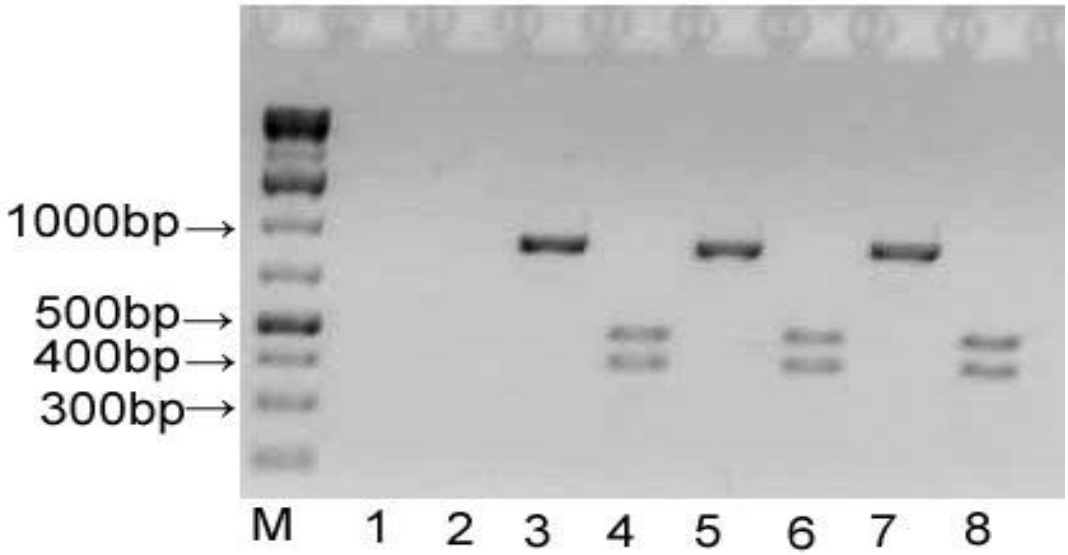

Figure 3.1 RT-PCR products of complete HVX CP gene, 850bp in size, using primers HVXs5639 and HVXa6488 (lanes 3, 5, 7) followed by restriction digest with *Hind* II into fragments 458 and 392bp (lanes 4, 6, 8). Each sample represents HVX isolate from each geographical region of Tennessee. Isolate Jn-1 from Johnson (lane3), Na-1 from Nashville (lane 5) and Ja-7 from Jackson (lane 7). Samples from a PCR product without any cDNA template was loaded in lane 1 and PCR product derived from RNA extracted from a HVX-free hosta was loaded in lane 2 where both served as negative control. Molecular markers were loaded in Lane M.

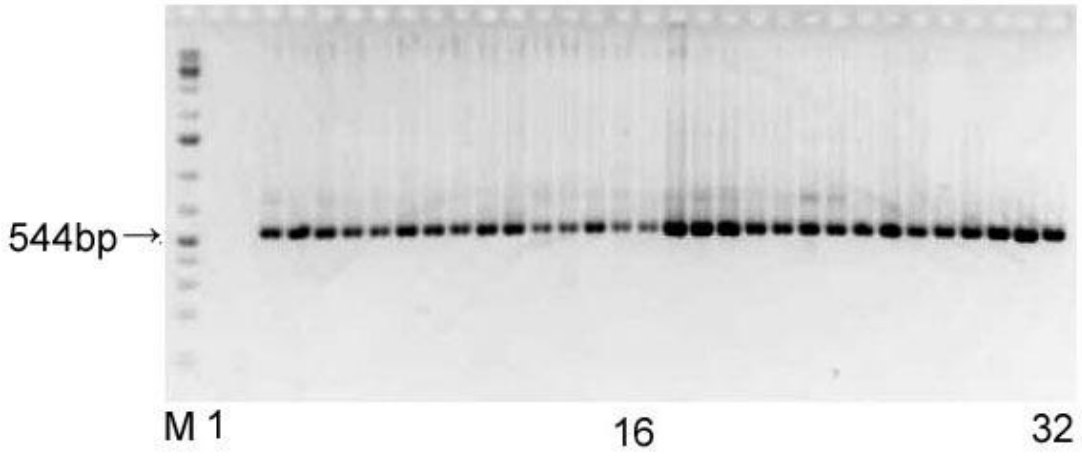

Figure 3.2 RT-PCR Products of partial CP gene of all TN isolates generated using conserved primers HVXs55 and HVXa598. The size of PCR products was 544bp. Lanes 3-9 contain samples from eastern, lanes 10-20 were loaded with samples from middle and lanes 21-32 are samples from western regions of Tennessee. PCR product without any cDNA template was loaded in lane 1 and PCR product with RNA extract derived from a HVX-free hosta was loaded into lane 2 where both serve as negative controls. Molecular markers were loaded in lane M.

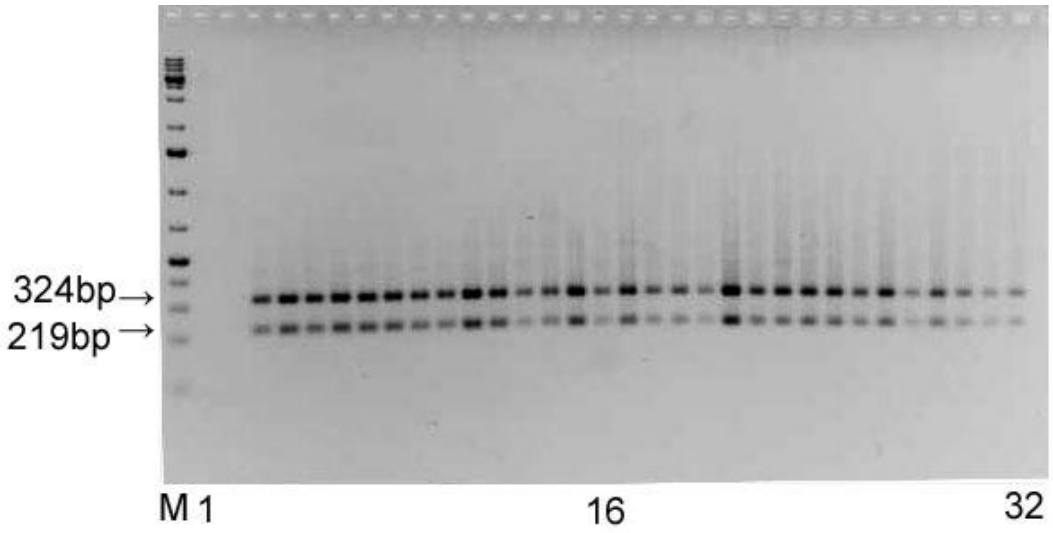

Figure 3.3 RFLP following RT-PCR derived from all TN isolates using *Hind* II restriction enzyme (lane 3-32). Fragments sizes of 219bp and 324bp were generated. Lanes 1 and 2 are negative controls where PCR was done in the absence of any cDNA template or cDNA template from a HVX-free hosta respectively. Lane M was loaded with the molecular makers.

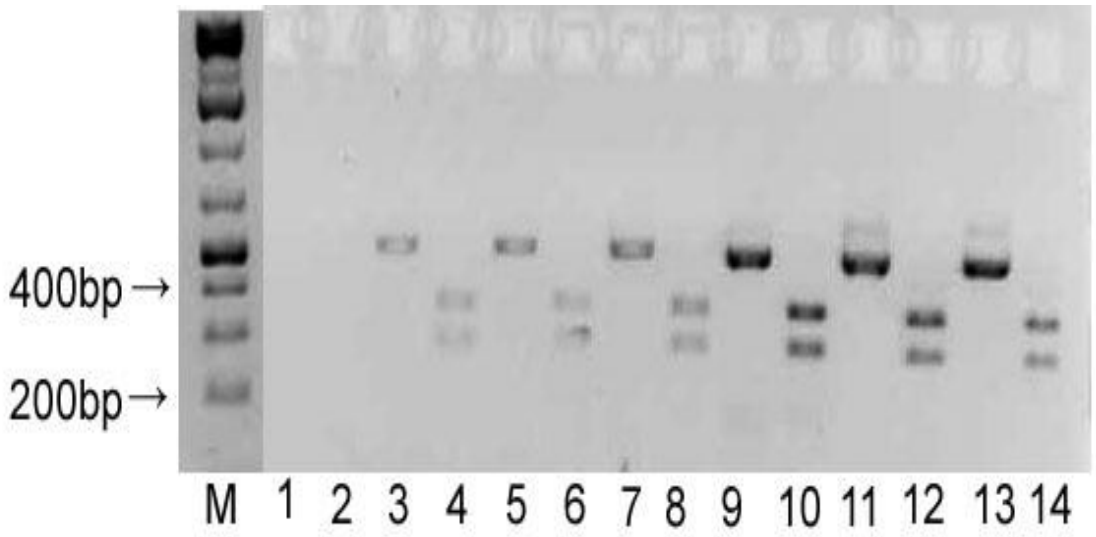

Figure 3.4 Sensitivity of RT-PCR-RFLP for detection of HVX in composite samples using HVX isolate Na-5 from hosta cultivar 'Yellow Splash' as source of infection. RNA templates extracted from mixtures of infected: HVX-free hosta leaf discs at ratios of 1:80, 1:40, 1:20, 1:10, 1:5, 1:0 were used to generate PCR amplicons of 544bp (lanes 3, 5, 7, 9, 11, 13). RT-PCR was performed with primers HVXs55 and HVXa598. RFLP following RT-PCR with *Hind* II produced fragments of 219bp and 324bp (lane 4, 6, 8, 10, 12, 14). PCR product derived from HVX-free hosta was loaded in lane 1 and followed by restriction digest (lane 2). Molecular weight markers were loaded in lane M.

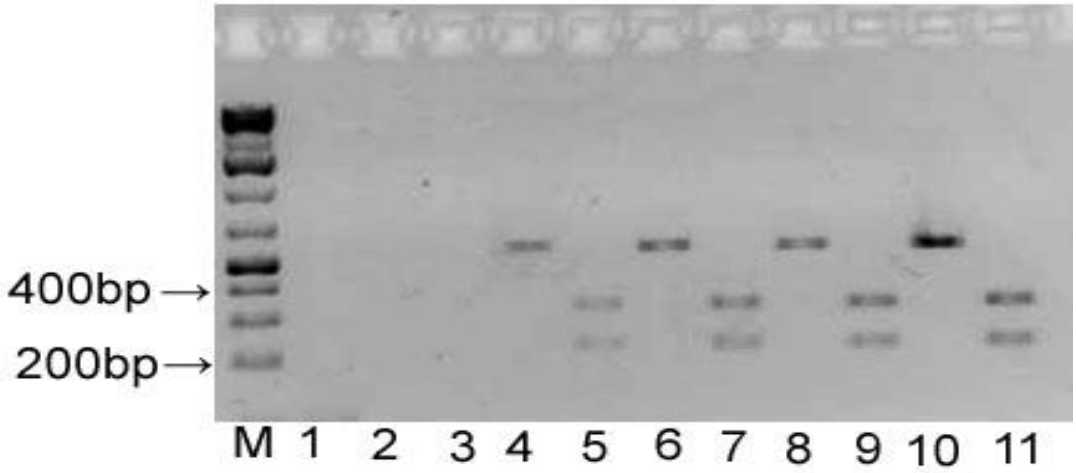

Figure 3.5 Evaluation of RT-PCR-RFLP for HVX detection in composite samples using hosta cultivar 'So Sweet' containing Mp-3 HVX isolate as source of infected leaf tissues. RNA templates extracted from mixtures of infected: HVX-free hosta leaf discs at ratios of 1:100, 1:40, 1:20, 1:0 were used to generate PCR amplicons of 544bp (lanes 4, 6, 8, 10) and followed by digestion with *Hind* II into smaller fragments of 219bp and 324bp (lanes 5, 7, 9, 11). PCR product derived from HVX-free hosta was loaded in lane 2 and followed by restriction digest (lane 3). Sample from PCR without any cDNA template was loaded in lane 1 and molecular weight markers in lane M.

Vitae

Oluseyi Lydia Fajolu (formerly Adedire) was born in Ado-Ekiti, Nigeria. She graduated from the Federal University of Technology Akure (FUTA), Nigeria with a first class honors degree in Microbiology. She was honored as the best graduating student of her class and also as the best graduating female student in the entire faculty of sciences. In 2005, she began her career in Academics as one of the pioneer staff of the department of Biological Sciences, Redeemer University, Nigeria, while she was also studying at FUTA for a Master of Science degree in Food Microbiology.

In 2007, she began another Master of Science degree program in Plant Pathology at the University of Tennessee, Knoxville (UTK), USA. She also started working as a Graduate Research Assistant at the Plant Virology Laboratory under the supervision of Dr. Reza Hajimorad. Her major research was on the Genetic Variability of *Hosta Virus X* in Hostas. Oluseyi was recommended in 2008 for the membership of the Honor Society of Agriculture, Gamma Sigma Delta. This was in recognition of her high scholarship and outstanding achievements. She is the first author of a paper titled: "*Hosta Virus X* in Hosta identified in Tennessee, USA" published in the Plant Pathology. She is currently finishing two additional papers from her thesis.

Oluseyi intends to pursue a Ph.D. at the Entomology and Plant Pathology Department of UT upon graduation. She currently lives with her husband Olufemi Nelson Fajolu in Knoxville and they have one daughter named Toluwani Miracle Fajolu.