The dissertation of Bin Tian was reviewed and approved* by the following:

Frederick G. Gildow  
Professor of Plant Pathology  
Head of the Department of Plant Pathology  
Chair of Committee  
Dissertation Co-Advisor

William L. Schneider  
Research Scientist of USDA-ARS  
Dissertation Co-Advisor  
Special Member

Gary W. Moorman  
Professor of Plant Pathology

Diana Cox-Foster  
Professor of Entomology

Stewart M. Gray  
Professor of Plant Pathology

*Signatures are on file in the Graduate School
Soybean Dwarf Virus (SbDV), a persistently aphid-transmitted luteovirus, is an important plant pathogen causing economic losses on soybean crops in Asia. In North America, indigenous strains of SbDV are commonly infecting clover, but have not emerged as a threat to soybean production. Failure of SbDV to spread from clover to soybean was believed due to the fact that SbDV was poorly vectored by *Aphis glycines*, the only aphid that colonizes soybeans. However, there are multiple isolates of SbDV in the United States that are *A. glycines*-transmissible. In order to evaluate the risk of SbDV as an emerging plant pathogen capable of threatening soybean production, the clover isolate SbDV-MD6 was serially transmitted from clover to pea or soybean by different aphid vectors, *Acrythosipon pisum*, *Nearctaphis bakeri*, and *A. glycines*. Virus titer, symptom severity, and transmission efficiency were evaluated for each passage. Sequence analyses of SbDV-MD6 from both pea and soybean passages identified 6 non-synonymous consistent mutations in pea, compared to 11 and 16 mutations in soybean when transmitted by *N. bakeri* and *A. glycines*, respectively. The \( d_N/d_S \) analysis indicated that SbDV was under strong selective pressures in soybean, but not in pea. Significantly increasing virus titers with each sequential transmission supports this analysis. However, aphid transmission efficiency on soybean decreased with each passage from 53% to 0%, until the virus was no longer aphid transmissible. Although virus titers increased, serial transmission by *A. glycines* ceased after only one or two passages in soybean. Results indicated that the clover strain of SbDV-MD6 adapted readily to soybean by improved replication and/or movement, but selection for host adaptation created tradeoff factors decreasing host-to-host transmissibility by aphid vectors. This may explain the reason SbDV-MD6 could not be sequentially transmitted by certain aphid vectors. In addition, to explain the cellular regulation mechanism of the aphid vector transmission, two isolates, SbDV-MD16 and SbDV-MD6, were used in the transmission assays. Virus titers in different aphids were examined by RT-qPCR during each passage. All aphids are able to acquire the virus even after 2 days of non-host feeding. Virus titers in aphids that fed on infected soybeans decreased with each sequential transmission suggesting that the
interaction between the virus and the aphid is a major bottleneck for the spread of SbDV-MD6. It is suggested that the most probable site is at the accessory saliva gland that is known for specific interaction between aphid and luteovirus in many systems. The selection on SbDV in soybean is probably leading to loss of specificity in this interaction. This may explain why clover strains of SbDV have not yet caused a severe epidemic in soybeans in N. America. Finally, SbDV-MD6 population diversity was examined in different host species. The diversity of SbDV populations increased slightly when at the beginning of shifting to a new host, and was relatively constant over serial passages. It also provided evidences that SbDV has the potential of expanding into a new niche and thus poses a threat of emerging as a new crop pathogen. However, the bottleneck imposed by persistent aphid transmission is the major limitation of SbDV spread in nature.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................ vi

LIST OF TABLES .......................................................................................................... vii

CHAPTER 1: Introduction ................................................................................................. 1
  General background .................................................................................................. 1
  The Barrier of Vector Transmission and Vector Specificity ................................... 3
  Viral Genomic Mutation Related to Viral Evolution and Host Adaptation .......... 9

CHAPTER 2: Host Adaptation of Soybean Dwarf Virus Following Serial Passages on Pea (*Pisum sativum*) and Soybean (*Glycine max*) ......................................................... 13
  Abstract .................................................................................................................. 13
  Introduction ............................................................................................................ 13
  Materials and Methods .......................................................................................... 15
  Results .................................................................................................................... 18
  Discussion .............................................................................................................. 23

CHAPTER 3: Cell regulation of interaction between Soybean Dwarf Virus and aphid vectors ........................................................................................................... 35
  Abstract .................................................................................................................. 35
  Introduction ............................................................................................................ 35
  Materials and Methods .......................................................................................... 38
  Results .................................................................................................................... 40
  Discussion .............................................................................................................. 42

CHAPTER 4: Determining the genetic diversity of Soybean Dwarf Virus in multiple host plants ........................................................................................................ 49
  Abstract .................................................................................................................. 49
  Introduction ............................................................................................................ 49
  Materials and Methods .......................................................................................... 51
  Results .................................................................................................................... 53
  Discussion .............................................................................................................. 55

CHAPTER 5: Conclusions and Future Directions ............................................................. 64

REFERENCES .............................................................................................................. 67
LIST OF FIGURES

Figure 2.1 Experimental design for the serial transmission of SbDV-MD6 in pea or soybean plants .................................................................28

Figure 2.2 Sequencing strategy for determination of full length consensus sequences of SbDV-MD6 ..................................................................29

Figure 2.3 Comparison of healthy and SbDV-MD6 infected pea and soybean..........30

Figure 2.4 Location of mutations that occurred on pea and soybean during serial transmission ........................................................................31

Figure 2.5 Real time RT-PCR virus quantification of SbDV-MD6 during serial transmission ........................................................................32

Figure 4.1 Genetic Diversity in a single plant Increased after reaching equilibrium......62

Figure 4.2 Distribution of accumulated mutations observed in clones derived from SbDV-MD6 populations ..............................................................63
LIST OF TABLES

Table 2.1 The transmission efficiency of SbDV-MD6 on pea and soybean .............33

Table 2.2 The transmission efficiency of back-inoculating clover with SbDV-MD6 ....34

Table 3.1 Transmission Efficiency of SbDV-MD6 on pea and soybean.................46

Table 3.2 acquisition and retention of SbDV-MD6 through serial passages.........47

Table 3.3 Relative concentration of soybean Dwarf Virus in the aphid vectors........48

Table 4.1 Genetic variation in SbDV-MD6 populations in different host species and passages.................................................................59

Table 4.2 Types of mutations observed in SbDV-MD6 populations in different host species and passages.........................................................60

Table 4.3 Substitution types.................................................................................61
CHAPTER 1

General background

The family *Luteoviridae* is one of the most important groups of plant viruses. They are responsible for high economic losses of important crops and have some unusual biological and molecular properties with simple structures and a relatively complex biology. The *Luteoviridae* consists of sixteen viruses in three genera: *Luteovirus*, *Polerovirus*, and *Enamovirus*. There are approximately 9 unclassified members and 17 tentative members (Herrbach, 1999; ICTV, 2008). In this dissertation, viruses in the family *Luteoviridae* are all referred to as luteoviruses for convenience. The virions of luteoviruses are simple, non-enveloped and isometric particles approximately 25-30 nm in diameter with icosahedral symmetry. A single stranded, positive sense RNA approximately 6 kb in size is encapsidated by the coat protein and the readthrough protein (RTP) (Gray and Gildow, 2003). Luteoviruses use several different translational strategies, including frameshifting, readthrough, leaky scanning, and internal initiation to regulate gene expression, and to enhance the information-carrying capacity of their relatively small genomic RNA (Gray and Gildow, 2003).

Symptoms in the host plants infected by luteoviruses include rolling, reddening or yellowing of leaves and stunting of growth. Luteoviruses are host tissue specific, and infect and replicate mainly in phloem tissue resulting in phloem necrosis. The host ranges of individual luteoviruses tend to be confined to one botanical family, except for Beet Western Yellows Virus (BWYV). The radish yellows strains of BWYV are able to infect at least 11 botanical families (Duffus, 1960). Each luteovirus species is transmitted only by a limited number of aphid species in the persistent circulative (non-propagative) manner displaying a high level of vector-specificity (Gray and Banerjee, 1999).

Soybean Dwarf Virus (SbDV) is a member of family *Luteoviridae*. Previously, SbDV was unclassified in the family *Luteoviridae*, because the nucleotide sequence of the 5’ region of SbDV resembles the genus *Luteovirus*; whereas, the 3’ region resembles the genus *Polerovirus*. Recently, SbDV has been assigned to the genus *Luteovirus* based on its genome organization and replication-related proteins (Fauquet et al., 2005).

Currently, four distinct phenotypes of SbDV (YS, YP, DS and DP) have been documented in Japan based on symptomatology in infected soybean and aphid vector...
specificity (Yamagishi et al., 2006). SbDV-YS and SbDV-YP are yellowing strains and cause severe interveinal chlorosis, rugosity and thickening of leaves in soybeans. SbDV-DS and SbDV-DP are dwarfing strains and cause stunting with shortened internodes and brittle curled leaves. The YS and DS strains are transmitted specifically by the aphid Aulacorthum solani, while YP and DP strains are transmitted by both Acyrthosiphon pisum and Neocerataphis bakeri. The subterranean clover red leaf virus (SCRLV), now considered as an Australasian (Australia and New Zealand) strain of SbDV (Rathjen et al., 1994), is transmitted by either A. solani or A. pisum. More recently, researchers in Germany reported two SbDV isolates infecting faba bean crops (Vicia faba L.) in Hebenshausen, and a SbDV-D strain in red clovers (Trifolium pratense L.) in Braunschweig. In addition, they discovered an unusual white clover (T. repens L.) isolate only transmitted successfully by A. pisum from clover to faba bean (Abraham et al., 2007). In the United States, two strains of SbDV, dwarfing (SbDV-D) and yellowing (SbDV-Y), are endemic primarily in clover plants and rarely infect soybeans (Damsteegt et al., 1990; Wang et al., 2006). A SbDV-like (SCRLV-like) virus was first isolated from legumes in California in 1983 and was specifically transmitted by A. pisum (Johnstone et al., 1984). Subsequently, it was reported that SbDV-like isolates naturally infected asymptomatic white clover, symptomatic red clover, yellow sweet clover (Melilotus officinalis), and subterranean clover (T. subterraneum L.) from 11 midwestern and eastern states in the U.S. (Damsteegt et al., 1999). In 2000, it was reported that soybean (Glycine max L Merr.) crops were infected by a yellowing strain of SbDV in Virginia (Fayad, 2000). During 2003, dwarfing strains of SbDV were found in soybean plants accompanied by the soybean aphid, Aphis glycines Matsumura, in Wisconsin (Phibbs et al., 2004). However, there are no data about vector relations among infections from this outbreak. In 2006, a survey of soybean diseases in northern Illinois also identified two dwarfing strains of SbDV (Harrison et al., 2005). In the United States, SbDV does not cause severe disease in soybean crops. The failure of SbDV to become epidemic in soybean crops in the U.S. is presumably due to the absence of efficient aphid vectors that colonize soybeans. However, a recent report indicated that some U.S. isolates of SbDV could be transmitted occasionally by A. glycines (Damsteegt et al., 2011). So far, little is known about the potential risk of indigenous clover-infecting strains of SbDV adapting to
a new aphid vector and becoming epidemic in soybean crops.

The Barrier of Vector Transmission and Vector Specificity
Luteoviruses are solely transmitted by aphids, one of the most crucial and most conserved features of luteoviruses. Aphids are natural vectors of many plant viruses including luteoviruses (D'Arcy and Nault, 1982). Luteoviruses are transmitted by aphids in a persistent non-propagative manner, often for the aphid’s entire lifespan (Gray and Banerjee, 1999). Luteovirus particles are acquired by aphids successfully feeding on phloem sieve elements or companion cells of virus-infected plants. The virus then moves through the aphid’s alimentary canal into the lumen of the aphid’s alimentary canal or gut (Bing et al., 1991; Gildow, 1993). Then the virus enters the epithelial cells of the aphid’s gut by endocytosis and is released into the hemocoel by exocytosis (Garret et al., 1996). Ultrastructural evidence (Gildow, 1999) suggest that in order for the virus to be acquired into the aphid’s body cavity or hemocoel, the virions must bind to virus-specific receptors on the gut apical plasmalemma of the epithelial cell. This binding mechanism initiates coated pit formation and endocytosis of the virus into the cell through coated vesicles. The virions are then transported by these clathrin-coated vesicles into the receptosome where virions are concentrated. Tubular shaped vesicles containing linear aggregates of virions bud off from the receptosome migrate toward the cell’s basal plasmalemma adjacent to the hemocoel, and release virions out of the cytoplasm (Gildow, 1985, 1993, 1999). These virions usually can diffuse through the gut basal lamina, a layer of extracellular matrix that is secreted by and surrounding the gut, and into the hemocoel. Based on in vitro and molecular studies (VandenHeuvel et al., 1997; VandenHeuvel et al., 1999), it has been hypothesized that luteovirus virions once released into the hemocoel, may bind to symbionin proteins which are GroEL-like proteins produced by the endosymbiotic bacteria of the genus Buchnera. The role of symbionins in the luteovirus transmission process, if any, is unclear, but it has been hypothesized that symbionin may protect virions from attack by the insect immune system, or may facilitate virions movement into the accessory salivary gland (ASG) (Hogenhout et al., 1996; Vandenheuvel et al., 1994). However, a recent study suggests that GroEL-like proteins from Buchnera are not sufficient to be detected in the hemocoel of A. pisum and R. padi,
and are unlikely to contribute to aphid transmission (Bouvaine et al., 2011). The contrary conclusions may result from experimental approaches in different systems. When virions suspended in hemolymph in the hemocoel do contact the ASG, virus-vector specific interaction occurs both on the basal lamina surrounding the gland and on the basal plasmalemma surrounding each accessory gland cell. Depending on the aphid biotype and the specific luteovirus, the virion may be blocked at the basal lamina or may diffuse through to the basal plasmalemma (Gildow et al., 2000; Peiffer et al., 1997). If the virions are recognized at the plasmalemma, they bind to virus-specific receptors and are endocytosed and accumulated into tubular vesicles in the cytoplasm. Coated vesicles containing individual virions pinch off from tubular vesicles adjacent to microvilli-lined canals (Gildow and Gray, 1993). These coated vesicles fuse with apical plasmalemma on the microvilli, forming coated pits to release virus into the salivary canal lumen, and virus is introduced back into the plant during aphid feeding (Gildow, 1999).

Two types of barriers to the persistent non-propagative transmission of luteoviruses in their vectors have been identified: gut barriers and salivary gland barriers. Virus ingestion into the alimentary canal of aphids begins with the penetration of aphid stylets into virus-infected phloem sieve elements or companion cells. This process is not specific and non-transmissible viruses will exit the aphid gut in the honeydew excrement (Gildow, 1993). The first barrier then encountered by a luteovirus is the midgut or hindgut. Generally, the gut does not play a critical role in specificity and many luteoviruses can penetrate the gut barrier of some common vector species of aphids and move into the hemolymph by the receptor mediated endocytotic/exocytotic process. Evidence of luteovirus recognition regulating virus acquisition into the hemocoel was indicated in one study that Cereal Yellow Dwarf Virus isolate-RPV could not get through the gut barrier of the non-vector *Metopolophium dirhodum* (Gildow, 1993). Other studies showed that *Myzus persicae* could acquire both Potato Leaf Roll Virus and SbDV, but through different cells of the midgut and the hindgut, respectively (Garret et al., 1993; Gildow et al., 2000). These observations indicate that the gut is the first recognizing site for virus acquisition, and the selective specificity is determined by different luteovirus-recognizing receptors in different regions of the gut (Garret et al., 1993; Gildow, 1993, 1999).

Luteoviruses in the hemocoel cannot be transmitted until they contact the ASG
where two barriers have been identified: the basal lamina that consists of a complex extracellular matrix of collegan and other glycoproteins surrounding ASG cells and the cell membrane (basal plasmalemma) of the ASG. The attachment of Barley Yellow Dwarf Virus isolate-MAV (BYDV-MAV) to the basal lamina was blocked when fab fragments of the monoclonal anti-BYDV-MAV immunoglobulin G were injected into the vector aphid, *Sitobion avenae*. The relatively small fab fragments bound to the MAV capsid and prevented virus attachment to and diffusion through the basal lamina and subsequent uptake into the gland. This suggested that virus capsid recognition and interaction with basal lamina surrounding the ASG might be important for vector specificity (Gildow and Gray, 1993). Furthermore, the importance of luteovirus and basal lamina interaction was clearly identified by comparing the ability of three BYDV vector-specific isolates to penetrate the basal lamina of five aphid vector species. When the dissected ASG of *S. avenae*, an efficient vector for BYDV-MAV, and *Rhopalosiphum maidis*, a non-vector, were incubated with purified BYDV-MAV, virions were consistently observed embedded in the basal lamina of *S. avenae*, but not in the basal lamina of *R. maidis*. The study also found that transmission efficiency of an aphid species might be partially regulated by the affinity of the virus for molecules on the ASG basal lamina, the size limit of the basal lamina pores, and net electrical charge of the basal lamina (Peiffer et al., 1997). Study of SbDV-Va20, another SbDV isolate in the U.S., indicated that a major barrier for SbDV also occurred on ASG cells, and that the vector selective specificity was recognized at both basal lamina and basal plasmalemma independently. SbDV-Va20 penetrated the salivary basal lamina of the non-vector *A. solani*, but was not observed in salivary gland cell; whereas, the Japanese strain SbDV-D was never observed penetrating through the salivary basal lamina of the non-vector, *M. persicae* (Gildow et al., 2000). Another example is the electron microscopy observation of CYDV-RPV accumulating at the basal plasmalemma of *S. avenae*, but never endocytosing in the cytoplasm of the ASG cells, thus never becoming transmissible (Gildow and Rochow, 1980). All these observations suggested a selective specificity system in luteovirus-aphids interaction, where the ASG basal lamina acted as a preliminary barrier filter, following by the cell plasmalemma as a second selective barrier.

Taken together, the selective specificity of the luteovirus transmission process seems
to occur at a minimum of three cellular sites, including the gut cell membrane, and the cell membrane and basal lamina of the ASG. These specific recognition sites are likely determined by multiple protein domains on the virus capsid and multiple cell surface receptors of aphid vectors.

The role of the viral coat protein in the virus transmission process is less clear. Luteovirus coat proteins are multifunctional proteins, involved in a variety of interaction activities such as virus replication, virus movement in plants, the interaction with vectors, and virion stability (Waigmann et al., 2004). It is accepted that virions assembled from only coat proteins are not transmissible when fed to aphids or microinjected into aphids, although they were detected at a low level in the hemolymph of aphids. For example, BYDV-PAV13 a mutant with a truncated form of the structural readthrough protein (RTP) was detected in the hemocoel of R. padi, but was not transmitted into plants and did not infect plants. Viral RNA from the PAV23 mutant which is unable to translate coat proteins, was not detected from hemolymph if injected into the aphid’s hemocoel, indicating that the coat protein is essential for virion RNA stability (Chay et al., 1996b). In the study of RTD-minus mutants of Beet Western Yellow Virus (BWYV-6.4), low concentration of viruses were detected in hemocoel, but not near or in ASG cells of the BWYV-vector aphid, M. persicae (Reinbold et al., 2001). This work provides indirect evidence that RTP was not required for the passage through aphid hindgut cells into hemocoel, but was apparently needed for virus transmission through the ASG. This observation is also consistent with the previously mentioned fact that the gut rarely contributes to the vector specific transmission process of luteoviruses (Gray and Gildow, 2003). It has been shown (Kaplan et al., 2007) that a single point mutation in the coat protein of PLRV caused great reduction of the transmission efficiency by the vector aphid, M. persicae; however, no further evidence explained which step was blocked in the virus circulative pathway.

Studies suggest that RTP may play an important role in luteovirus vector-specificity interactions. The RTP containing a highly conserved N-terminal region and a variable C-terminal region is obviously a multifunctional protein as well. Transmission assays with a set of BWYV deletion mutants in the RTD showed that the N-terminal conserved region of RTP had important effects on aphid transmission (Bruyere et al., 1997). N-terminal
RTD deletion mutations on BWYV resulted in no binding to symbionins and less stable virions in the hemocoel in the aphid vector, *M. persicae* (VandenHeuvel et al., 1997). In contrast, purified BYDV-PAV with C-terminal RTD truncations were still efficiently transmitted by *R. padi* (Wang et al., 1995). These observations reveal that RTP, especially the N-terminal region, is likely crucial for virus recognition at the ASG level. In addition, the RTP also appears to govern the gut recognition and transport efficiency. Study of BWYV-5.123L mutant, with a three amino acid change in the central part of the RTP, showed that virions were observed embedded in the basal lamina of the midgut cells, and still could be transmitted by *M. persicae* with reduced frequencies (Brault et al., 2007; Brault et al., 2000b). The exchange of RTD encoding sequences between BWYV and Cucurbit Aphid-borne Yellows Virus (CABYV) caused an inversion of vector specificity, and a modification of gut tissue tropism. Brault et al. (2007) constructed two hybrid viruses, BW-RTDCA and CA-RTDBW by exchanging the ORF5 sequences between BWYV and CABYV. BW-RTDCA can be transmitted by *A. gossypii*, which is a vector of CABYV; whereas, BWYV and CA-RTDBW were not transmissible. Moreover, BW-RTDCA was visualized under transmission EM, like CABYV transporting through both mid and hindgut of *M. persicae*; whereas, CA-RTDBW was only observed in midgut cells, like BWYV (Brault et al., 2005). In a recent study of the mutagenesis of PLRV RTP, none of the three-amino-acid N-terminal deletion or the non-incorporated RTD mutants were transmissible by *M. persicae* (Peter et al., 2008). Moreover, RTP also plays a role in virus accumulation in plants (Brault et al., 2000b; Chay et al., 1996b).

Phylogenetic analysis of the complete nucleotide sequences of four Japanese SbDV isolates showed that sequence identity was highest between isolates with the same symptomatology. Though the C-terminal portion of the RTD were very different between Y and D strains, the N-terminal portion of the RTD were very similar between YS and DS and between YP and DP (Terauchi et al., 2001). This is consistent with the suggestion that the N-terminal portion of the RTD is likely closely involved in aphid vector specificity of SbDV. However, the major determinant sequence for aphid transmission in N-terminal portion of the RTD has not been identified. Furthermore, it was speculated that SbDV acquired the four genotypes by evolutionary events probably resulting from either recombination between the ancestral Y and D strains, or accumulation of mutations and
strong selection pressures for vector specificity (Terauchi et al., 2003).

Several luteovirus-binding aphid proteins, including potential cell receptors, have been identified. For instance, Rack-1 homologue in *M. persicae*, had clear interaction with the RTP of BWYV-WT *in vitro*, but not with BWYV-6.4 and -5.123L mutants that lack the RTP and display altered efficiency in aphid transmission (Reinbold et al., 2001). Rack-1 is a conserved multifunctional protein important in regulating cell surface receptors and some kinases in all plants, animals, and invertebrates (Choi et al., 2003). It is suggested that Rack-1 might be a critical element in the endocytosis/transcytosis process (Seddas et al., 2004). In addition, GAPDH3 in *M. persicae* was hypothesized to be a receptor for BWYV in aphid midgut and ASG cells, based on the homologues in other organisms regulating the actin-dependent endocytosis and exocytosis (Seddas et al., 2004). BYDY-MAV bound to two proteins of the aphid vector *S. avenae*, SaM35 and SaM50, in virus overlay assays, but these same proteins were not present in the non-vector aphid *R. maidis* (Li et al., 2001). It was suggested that SaM35 and SaM50 isolated from head tissues might be receptors in MAV transmission based on their high affinity for MAV binding to the efficient vector, *S. avenae*. Genetic analysis by sexual crossing between *Schizaphis graminum*-F and *S. graminum*-SC, which differ in the ability to transmit CYDV and BYDV, indicated that genetic inheritance of vector transmission efficiency was regulated by a few major genes and several minor genes that function in an additive manner (Burrows et al., 2007). Furthermore, proteome analysis showed that four proteins specifically binding to CYDV-RPV in the vector aphid *S. graminum* were inherited and conserved in different generations of vector genotypes (Yang et al., 2008). Two of these proteins were identified as luciferase and cyclophilin, both involved in macromolecular transport in cells (Oba et al., 2005; Pemberton and Kay, 2005). Co-immunoprecipitation of aphid proteins and RPV provided evidence that these proteins bind CYDV-RPV and are likely involved in virus transmission process, though their specific functions still remain to be examined.

These results showed that luteoviruses required different interactions with different vector aphid proteins in order to be transported through the gut and the ASG transmission barriers. The direct evidence for the luteovirus-vector specificity occurring at the ASG site was that BYDV-RPV particles were observed to accumulate between the ASG basal
lamina and the plasmalemma, but lacked the ability to penetrate through the plasmalemma or cell membrane and accumulate in the ASG of non-vector, *S. avenae* (Gildow and Rochow, 1980). It is hypothesized that luteoviruses utilizing different capsid protein domains interact with different receptors in aphids to move through the gut and the ASG barriers (Gildow, 1999). With more genetic factors and molecular mechanisms to be understood, the reason for luteoviruses having limited and specific aphid vectors remains unknown and is worth further study.

**Viral Genomic Mutation Related to Viral Evolution and Host Adaptation**

Plant viruses have several mechanisms to generate genetic diversity both within and between species. Plant RNA viruses have highly error prone replication mechanisms, which result in numerous mutations and a quasi-species nature. Mutation, recombination, and re-assortment are three major forces driving the evolution of viruses, and these forces generate diversity in viral genomes, providing variants with the ability to adapt to different environments (Roossinck, 1997). The mechanisms of RNA recombination and re-assortment have been documented (Chare and Holmes, 2006; Fargette et al., 2006; Ohshima et al., 2007). However, mechanisms of mutation and selection and how they contribute to virus adaptation and fitness are less well understood.

Change of the internal or external environment or survival niche of a virus may require different adaptive strategies for different plant viruses. Viruses that can infect and replicate in different plant species probably have different selection pressures in each host. It is known that both coding sequences and the non-translated regions of viruses have important functions in biological activities related to fitness and survival in the host. The vector transmission of viruses is also important factor for viral adaptation and evolution. The evidences from geminiviruses and potyviruses suggest that vector transmission imposes significant constraints on the evolutions of plant viruses (Power, 2000). Still little is known about viral adaptation to vectors.

The viral evolution for host adaptation based on mutation and selection is important because of its implication for the understanding of newly emerging infectious diseases. Generally speaking, plant viruses have variable host ranges: some infect only one or a few related species, while others can infect a wide range of hosts from different
taxonomic groups. It is known that the majority of plant viruses have limited host ranges (Schneider and Roossinck, 2000). Considering the potential advantages associated with being able to use multiple hosts, why are many viruses specialized in a few hosts? It has been suggested that fitness trade-offs would occur during the selective adaptation of alternative hosts, or because evolution would proceed faster within smaller host ranges (Alizon et al., 2009; Read, 1994). It is known that adaptation to a new host often results in fitness losses in original hosts because mutations beneficial in the new host might be detrimental to infection of the original hosts. This is called trade-off effect (Elena and Sanjuan, 2005). There is an increasing amount of evidence confirming the existence of this trade-off hypothesis (Anderson and May, 1982; Messenger et al., 1999). For instance, studies of Plum Pox Virus (PPV) indicated that different specific mutations responding to mechanical or aphid-transmission occurred associated with the PPV adaptation to a new host, pea (Pisum sativum L.) (Wallis et al., 2007). The interesting thing, however, was that there appeared to be a very limited trade-off on PPV fitness in the original peach host after adaptation to peas, along with the increasing the transmission efficiency. One study indicated that the site-specific mutation to amino acid 27 of NlaPro of Papaya Ringspot Virus (PRSV) determined the ability for infecting the host papaya (Mangrauthia et al., 2008). Another study using Soybean Mosaic virus (SbMV) showed that sequential transfer of SbMV with additional mutations resulted in adaptation to another genotype soybean. The precise mutations on HC-Pro are essential and sufficient for the virulence on different genotype soybeans (Hajimorad et al., 2008). In addition, Tobacco Etch Virus (TEV) adapted to a new host, pepper, as indicated by an increase in virulence and virus accumulation, the viral fitness in a natural host was decreased (Agudelo-Romero et al., 2008).

Multiple evolutionary processes affect evolution in the viral population: genetic drift and selection or genetic exchange (re-assortment and recombination) (Garcia-Arenal et al., 2003). The effects of genetic drift and selection are always closely related, because the population may also increase the diversity if under different selection pressure (Altschuh et al., 1987). Viral selection can be associated with any factor, such as ability to infect and replicate in host plants, ability to be transmitted by their vectors, and maintenance of functional protein structures, such as components of the protective coat
protein needed for maintaining RNA viability. The strongest selection factors are most likely those associated with the host adaptation, due to the fact that this is the only environment where plant viruses actively replicate. A study of genetic diversity of BYDV-PAV isolates in a natural population showed that the host species was the main contributor to PAV population diversity (Mastari et al., 1998). In addition, more evidence for host adaptive selection comes from the phenomenon of viral overcoming of host resistance genes (Garcia-Arenal and McDonald, 2003). A good example is the loss of Tm1 gene resistance against Tomato Mosaic Virus (ToMV) in tomato cultivars. A single nucleotide change in the ToMV 126k protein (His984-Tyr) was enough to convert ToMV into a Tm1 resistance-breaking isolate (Meshi et al., 1988). There also are examples for the trade-off phenomena between increased virulence and decreased transmissibility as in the population of CMV and its satRNA. For example, the different variants of Cucumber Mosaic Virus (CMV) satellite RNAs derived from consistent selection in different plant hosts have been documented (Kaper et al., 1988; Kurath and Palukaitis, 1989). Studies found that transmission efficiency of CMV correlated positively with the titer of viruses in source plants, while the presence of CMV-satRNA reduced the transmission efficiency, but increased the disease virulence by increased necrosis (Escriu et al., 2000a; Escriu et al., 2003; Escriu et al., 2000b). Vector-associated virus selection has been documented as well. For example, studies suggested that Wound Tumor Virus, persistently propagatively transmitted by leafhoppers, lost the vector transmissibility after being repeatedly multiplying in non-vector passages (Reddy and Black, 1977). The aphid vector, *M. persicae*, also mediated the establishment of a genomic recombinant when probing on plants co-infected with the cucumoviruses, Tomato Aspermy Virus (V-TAV) and CMV. The aphid transmitted progeny viruses induced distinct symptoms differing from either parental virus, and had different RNA profiles (Perry and Francki, 1992). Other evidence of vector selection involved individual whiteflies acquiring more than one begomoviruses, and assisting in the production of pseudo-recombinants with the acquisition of additional DNA components coding for antigenic variants (Harrison and Robinson, 1999). However, to our knowledge, little evidence is found for luteoviruses evolution caused by mutation and natural selection by host-associated or vector-associated factors.
Clearly, extensive research about evolutionary strategies of plant viruses is required to provide comprehensive knowledge about how these simplest forms of life can master the adaptation and fitness to this ever-changing world.
CHAPTER 2
Host Adaptation of Soybean Dwarf Virus Following Serial Passages on Pea (*Pisum sativum*) and Soybean (*Glycine max*)

ABSTRACT
Soybean Dwarf Virus (SbDV) is an important plant pathogen, causing economic losses in soybean. In North America, indigenous strains of SbDV mainly infect clover, with occasional outbreaks in soybean. To evaluate the risk of a US clover strain of SbDV adapting to other plant hosts, the clover isolate SbDV-MD6 was serially transmitted to pea and soybean by aphid vectors. Sequence analysis of SbDV-MD6 from pea and soybean passages identified 11 non-synonymous mutations in soybean, and 6 mutations in pea. The \( d_s/d_D \) analysis indicated that SbDV was under positive selection in soybean, but not in pea. Increasing virus titers with each sequential transmission supported this analysis. However, aphid transmission efficiency on soybean decreased until the virus was no longer transmissible. Results indicated that the clover strain of SbDV-MD6 adapts to soybeans. However, mutations that improve replication and/or movement may have trade-off effects resulting in decreased vector transmission.

INTRODUCTION
Soybean Dwarf Virus (SbDV), first identified as a pathogen of soybean plants in northern Japan in 1969, is capable of causing serious yield losses in soybean (Tamada et al., 1969). The host range of SbDV is largely limited to members of the *Fabaceae* except for a few species in *Chenopodiaceae* and *Polemoniaceae* (Damsteegt et al., 1990). Currently, four distinct strains of SbDV are recognized in Japan based on sequencing, symptomatology in infected soybean plants and aphid vector specificity (Yamagishi et al., 2006). The yellowing strains, SbDV-YS and SbDV-YP, cause severe interveinal chlorosis, rugosity and thickening of leaves in soybeans. The dwarifying strains, SbDV-DS and SbDV-DP, cause stunting with shortened internodes and brittle curled leaves. The YS and DS strains are transmitted specifically by the aphid *Aulacorthum solani* Kaltenbach, while YP and DP strains are transmitted by both *Acyrthosiphon pisum* Harris, and *Nearctaphis bakeri* Cowen. In the United States, SbDV-like isolates were identified in asymptomatic white
clover (*Trifolium repens*), symptomatic red clover (*Trifolium pratense*), yellow sweet clover (*Melilotus officinalis*), and subterranean clover (*Trifolium subterraneum* L.) from 11 mid-western and eastern states (Damsteegt et al., 1999). In 2000, it was reported that soybean (*Glycine max* L Merr.) crops were infected by a SbDV-Y strain in Virginia (Fayad, 2000). During 2003, dwarfing strains of SbDV emerged in soybean plants accompanied by the soybean aphid, *Aphis glycines* Matsumura, in Wisconsin (Phibbs et al., 2004). A survey of soybean diseases in northern Illinois also identified two SbDV-D strains (Harrison et al., 2005). Recently, the presence of mixed infections of both D and Y strains were confirmed in the eastern United States (Schneider et al., 2011), and several U.S. isolates were found to be transmitted by *A. glycines* (Damsteegt et al, 2011).

SbDV is a luteovirus, with a positive sense RNA genome. The genomic RNAs of SbDV range from 5.7 to 5.9 kb, comprising five open reading frames (ORFs) and three untranslated regions (UTRs). ORF 1 and ORF 2 encode the replication-related proteins. ORF 3 encodes the 22-kiloDalton (kDa) major coat protein that is the major component of the capsid, and ORF 4, which is nested within ORF 3, putatively encodes a movement protein. ORF 5 encodes a 65-88 kDa Read-Through Protein (RTP), the minor capsid protein, formed by the in-frame translational readthrough of the ORF3 stop codon. ORFs 3, 4 and 5 on the 3′-end of SbDV show similarities to genus *Polerovirus*, while ORFs 1 and 2 show similarities to the genus *Luteovirus* (Rathjen et al., 1994; Terauchi et al., 2001).

Plant viruses have several mechanisms to generate genetic diversity both within and between species. Plant RNA viruses have highly error prone replication mechanisms, which result in numerous mutations and diverse populations. Mutation, recombination, and re-assortment are three major forces driving the evolution of viruses, and these forces generate diversity in viral genomes, providing variants to adapt different environments (Roossinck, 1997). Viral emergence and adaptation to new hosts and/or new host resistances is one of the highest impact effects of plant virus evolution (Garcia-Arenal and McDonald, 2003). Even minor changes in viral genomes can result in significant phenotypic effects. One study indicated that the site-specific mutant on amino acid 27 of NIaPro of Papaya Ringspot Virus (PRSV) determined the ability for infecting the host papaya (Mangrauthia et al., 2008). Another study on Soybean Mosaic virus (SbMV)
showed that precise mutations in the HC-Pro gene were essential for virulence on different resistant genotypes of soybeans (Hajimorad et al., 2008). However, there may be limitations to the amount of host adaptation a plant virus can tolerate. Adaptation to a new host could result in fitness losses in original hosts because mutations beneficial in the new host might be detrimental to infection of the original hosts, a phenomenon called trade-off effect (Elena and Sanjuan, 2005). For example, Tobacco Etch Virus (TEV) adapted to a new host, pepper, as indicated by an increase in virulence and virus accumulation; whereas, the viral fitness in the natural tobacco host was decreased (Agudelo-Romero et al., 2008). In the case of SbDV, a trade-off effect that increases fitness in soybeans while decreasing fitness in clover could have significant epidemiological impacts, as clover is the overwintering reservoir for the virus.

Reports indicate that SbDV is widely prevalent in clovers in North America (Damsteegt et al., 1995). There are currently only limited reports of SbDV emergence in soybean fields (Fayed et al., 2000; Phibbs et al., 2004), despite the high profile and acreage of this important crop. It is quite probable that SbDV is undergoing adaptation in the transition from clover to soybean. The risks of SbDV outbreaks in soybean would seem to be higher following the recent introduction of the soybean aphid (A. glycines), and the establishment of this potential SbDV-vector species on soybean crops. Therefore, a better understanding of the capacity for SbDV host adaptation is important to evaluate potential risk of SbDV epidemics in commercial soybean crops in the U.S. The objectives of this study are to evaluate the SbDV fitness in different plant hosts and identify critical mutations of SbDV selected by such new host adaptations.

**METHODS AND MATERIALS**

**Plants, viruses, and aphid vector**

For this study, Puget pea (Pisum sativum cv. Puget), soybeans (Glycine max cv. Williams), and white clover (Trifolium repens) seedlings were used for host serial transmissions. A white clover infected with a SbDV-Y isolate (MD6) was collected from a field survey in Maryland. SbDV-MD6 was maintained in white clover from the time of field collection until the beginning of the host passaging. In the aphid transmission
experiments, *A. pisum* (pea aphid) was used as the aphid vector for pea passages, and *N. bakeri* was used as the vector for soybean passages. Pea aphids were reared on caged faba bean plants (*Vicia faba* L.), and clover aphids were reared on red clovers (*Trifolium pratense* L.) in an aphid rearing room maintained at 25°C with 24 hours photoperiod.

**Serial transmission assays**

For the transmission experiment, aphids acquired viruses by feeding on detached leaves of infected white clover for 24 hrs. Then, the aphids were transferred to healthy pea or soybean seedlings for 5 days. At the same time, three healthy seedlings of each plant species were fed on by healthy aphids as a negative control. For *A. pisum* on peas, 3 aphids or 10 aphids per plant were used in the different serial transmission lines. For *N. bakeri* on soybeans, approximately 30 aphids per plant were used in all serial transmission lines. The number of aphids used per plant on each host species was determined by preliminary transmission efficiency tests. After inoculation, the percentage of infected plants was determined by ELISA according to manufacturer directions (Agdia, Elkhart, IN). Detached leaves from all infected plants were pooled to serve as the source for the next transmission (Figure 2.1). Each passage line was continued through eight passages, or until transmission failed. SbDV-MD6 was transmitted four times by both aphids in the original host, white clover, as a positive control for transmission, and as a control to determine whether subsequent effects were related to host shifts or merely to passaging. The transmission efficiency was analyzed by Chi-Squared test from Minitab 15 (Minitab Inc., State College, PA). For pea passage lines, all passages were compared with the first passage. For soybean passage, because there are not enough positive samples, no valid statistics could be done.

**Plant height and fresh weight measurements and statistical analysis**

To compare healthy and infected plants from various passages, plant height was measured at 25 days post-inoculation (d.p.i.). The fresh weight of pea stems and leaves was tested by weighing plants at 30 d.p.i. The statistical significance of differences in plant height and fresh weight between non-infected and infected plants, and among passages was subjected to *t*-test analysis from Minitab 15 (Minitab Inc., State College, PA).
RNA extraction and real time RT-PCR

In each transmission experiment, leaf samples from all infected plants were collected from every passage individually, and flash-frozen in liquid nitrogen and stored at -80°C for RNA extraction. The leaf tissue from each infected plant was randomly cut by a leaf punch with 1cm diameter to grind in tubes with liquid nitrogen, and 3 discs were used for extracting total RNA. Total RNA of samples was isolated using the RNeasy Mini Kit (Qiagen Science, Louisville, KY) according to manufacturer specifications, and diluted in 50µl buffer. SbDV RNA was amplified using first strand cDNA synthesis as follows: 2µl of total nucleic acid was added to 1x first strand buffer (Invitrogen, Carlsbad, CA), 10mM DTT, 300µM dNTP mix, 40U SuperScript II (Invitrogen), and 200nM SbDV reverse primers (Figure 2) to cover the full length of SbDV genome. The reaction was incubated at 42°C for 1 hour. Amplification followed using 1µl of the first strand reaction for the quantitative PCR (qPCR). The primers used in the qPCR assay were 1880UR (5’- cat tta ttg gct att atc ttc c -3’) and 1548YF (5’- caa caa agt tgg ttg tcc aag gac cc -3’), and the fluorescently labeled probe was 1728UP (5’- gat agc acc cag gtt gat atg t -3’). The reaction was run in a SmartCycler (Cepheid) machine with the following conditions: 10 min 95°C denaturing followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 min. The assay positive result threshold was calculated by the SmartCycler program using 10 standard deviations above background fluorescence. To determine the effectiveness of quantification, a fragment of SbDV-MD6 was cloned into a plasmid with a T7 RNA polymerase promoter. This plasmid was linearized and used to make in vitro transcripts, which served both as positive controls for qRT-PCR and as a directly quantifiable RNA template to establish standard curves. The number of the copies of SbDV-RNA was estimated by the formula \((pg \times 6.023 \times 10^6)/3.74\). The statistic analysis of virus titers was done with one way ANOVA, significant difference was tested using Fisher’s method.

Back inoculation assays

Various selected passages from the serial transmission on peas (Table 2.2) were used as a source for back inoculation assays, to test the ability of aphids to transmit the pea- or soybean-adapted SbDV back into the clover host. Only the first and the final soybean
passage was used as source for back inoculation assay. The two aphid vectors, *A. pisum* or *N. bakeri* were fed on infected pea or soybean leaves, respectively, for 24 hours, then transferred to healthy white clover seedlings. The pea and soybean passaged SbDV-MD6 was transmitted to 6 and 10 healthy clover plants respectively, with 6 plants for positive controls (*A. pisum* transmitting SbDV from pea to pea, and *N. bakeri* transmitting source SbDV from clover to clover). Infected plants were determined by ELISA. Samples were collected from positive plants, and the total RNA was isolated for sequencing.

**Sequencing and analysis**

To identify genomic mutations associated with the adaptation to new hosts, the complete genome of the final passage and one of the middle passages from each of the passage lines was sequenced and compared to the viral genome from the original clover isolate. Total RNA was extracted as previously described. RT-PCR products were generated for the final passages from each of the passage lines using specific primer pairs designed to cover the entire SbDV genome (Figure 2.2). The PCR products were directly sequenced with both directions to determine the consensus sequences (most common sequence). The consensus sequences were aligned with the original isolate from clover by using Clustal_X of the sequence analysis software Bioedit (Ibis Therapeutics, Carlsbad, CA). Consistent mutations (mutations that occurred in both passage lines, and were maintained through to the end of passaging) between the source isolates and alternative host passages were identified, and effects on amino acid content of proteins were determined. The back assay populations were sequenced using the same approach to look for reversion of the potential adaptive mutations. The $d_{SV}/d_S$ ratio was calculated using MEGA4.0 (Tamura et al., 2007) with Nei-Gojobori method.

**RESULTS**

The isolate used for this study was SbDV-MD6, a yellowing (Y-like) isolate collected from Prince George’s county in Maryland. The infected white clover was collected from the edge of a soybean field, but there was no symptoms observed of SbDV infections in the adjacent soybeans. Multiple infected white clovers were identified surrounding the soybean field. Both *A. pisum* and *N. bakeri* were present in the clover surrounding the
soybean fields, but neither of these aphids was observed feeding on soybeans. Based on this information, the natural history of SbDV-MD6 was assumed to be limited to a clover infection, transferred from clover to clover by *A. pisum* and/or *N. bakeri*. There were no peas (*Pisum sativum* L.) anywhere within a 1 km radius of the originally infected white clover.

**Serial passages and vector transmission efficiency on peas and soybeans**

To investigate the potential ability of SbDV to adapt to other alternative hosts, peas (cv. Puget) and soybeans (cv. William) were used as plant hosts for serial transmissions of SbDV-MD6 by *A. pisum* and *N. bakeri*, respectively. Different aphid species were used because of the aphid host selectivity, but clover to clover SbDV-MD6 transmission efficiency was roughly equal for both vector species (data not shown). The SbDV-MD6 isolate was maintained in the same white clover from the point of collection until the beginning of the passaging experiments, with additional infected source plants generated solely by the vegetative propagation of runners. This clover isolate was used as an initial inoculum source for pea and soybean lines, and these infected pea and soybean plants were then used as sources for serial transmissions, continuing through eight passages, or until transmission was lost. The transmission efficiency of each passage was determined by the percentage of successfully infected peas or soybeans.

In pea, initial SbDV-MD6 inoculations were done with 10 aphids for each pea seedling. The transmission efficiency was 67% at first passage, and remained fairly consistently above 80% after the second passage with the exception of passage 5 (Table 2.1). In a second serial transmission with 3 aphids per plant, improvement was observed after the third passage, and the transmission efficiency was maintained around 40% until the end of the serial passage experiment (Table 2.1). Based on the Chi-Square Test on the frequency of infected plants and non-infected plants, in pea serial passages, the transmission efficiency had a statistically significant increase with passages when using three pea aphids per plant. In contrast to serial passages in pea, SbDV-MD6 transmission efficiency in soybean generally deceased with serial transmission. The transmission efficiency on soybeans was more variable than the transmission efficiency on peas, and after five or six passages in soybeans, SbDV-MD6 lost transmission ability (Table 2.1).
Symptom development and virus titer in alternative hosts

The level of symptom development was determined by observing visible symptoms and by measuring plant height and fresh weight in each passage. The symptoms of SbDV-MD6 infected peas did not appear until at least 20 d.p.i. (data not shown). All infected pea plants from various passages (P1-P8) were significantly shorter and had less weight than the healthy controls (Figure 2.3A). Despite the fact that SbDV-MD6 is a yellowing strain, there was no more chlorosis in infected pea leaves than in healthy controls (Figure 2.3A). Plants in the final passage (P8) of SbDV-MD6 in pea were also significantly shorter, and had reduced fresh weight compared those of the first passage (Figure 2.3A). Therefore, symptom severity did increase slightly following serial transmissions in pea.

Symptoms on soybeans were less dramatic. Infected soybeans showed only moderate levels of chlorosis after 30 days when compared with healthy soybeans (Figure 2.3B-C). No significant dwarfing or reduced fresh weight was observed in soybean at any time (Figure 2.3C and data not shown). Symptom severity of SbDV-MD6 infected soybean did not increase with continued passages.

Quantitative real time RT-PCR was used to assess viral titers of SbDV-MD6 in pea and soybean at each passage (Figure 2.5). Virus titers were determined in each of two separate experiments (Line1 and Line2) for both pea and soybean. Using a SbDV-MD6 *in vitro* transcript as a template, the standard curve was established with serial dilutions in healthy plant extracts. The assay consistently detected less than 1 femtogram (fg) of SbDV RNA transcripts. Regression analysis of the SbDV standard curve demonstrated that the assay is highly efficient (R$^2$=0.998) indicating that it can be used to estimate SbDV-RNA concentration (data not shown). The mean titer of first passages in pea was 3.7x10^5 target molecules per nanogram (ng) of total RNA extraction. The first great increase in virus titers occurred between the first and second passages (2-fold), and virus titers of all pea treatments trended consistently higher values for P2-P6 except P5, but variation and standard deviation of replicates did not indicate a significant difference from P1. The virus titers of P7-P8, however, were significantly different from P1, suggesting increased SbDV concentration in peas following sequential transmission in the new pea host. Overall, in both passage experiments the titers of SbDV-MD6
increased slightly with continued passage on peas (Figure 2.5). The mean virus titer in the initial passages of soybean was 4.3x10^5 targets molecules per ng of total RNA extraction, and the virus titers for subsequent passages continued to increase, suggesting increasing virus titers with each subsequent transmission. The virus titers kept trending up to 2.6x10^6 targets (Soybean line 1) and 2.3x10^6 targets (Soybean line 2) at the last passages. Overall, there was a more dramatic significant increase of viral titers resulting from sequential soybean transmissions compared to pea. Based on the mean virus titer of line 1 and line 2 in the Figure 2.5, SbDV-MD6 had an approximately 6-fold increase in viral titer by the end of both serial passage experiments.

**Clover back-inoculation assays**

Pea and soybean adapted SbDV-MD6 were transmitted back to white clover to evaluate the potential trade-off effects associated with adaptation to new hosts. The back-inoculation transmission efficiency decreased with continued passage on the new host (Table 2.2). However, pea-adapted SbDV-MD6 populations never lost the ability to re-infect clover, even after 8 passages in pea. Back inoculations from soybean-adapted populations to healthy clover seedlings were only attempted after the first and final passages. The transmission efficiency was reduced greatly after SbDV-MD6 populations moved into the new soybean host. SbDV-MD6 in final passages (Table 2.2, passage 5 and passage 6) lost aphid transmission ability and serial transmission ceased on soybeans. The reduction in clover infection efficiency by soybean adapted SbDV-MD6 suggested that SbDV-MD6 populations encountered the reduced fitness in clover by either reduced transmission ability and/or reduced infection capacity in clover.

**Sequence analysis**

To determine what molecular changes had occurred in the viral population following the infection of new hosts, the complete genomes of SbDV-MD6 in pea and soybean passage lines were sequenced. First, the complete genome of original population in clover was sequenced as a reference for comparison. The completed consensus sequence of original white clover SbDV-MD6 included 5862 nucleotides, and the genome organization of the five major ORFs was consistent with all other SbdV-Y strains (Rathjen et al., 1994) (data
not shown). In the pea lines, the complete genome of first passage, two middle passages (P2, P4), and last passage (P8) were sequenced. The same approach was taken with the soybean lines. As a control, the last passage in the clover-to-clover transmission line, done in parallel with the pea lines as a positive transmission control, was also sequenced.

For serial transmission on peas by *A. pisum*, there were a total of 17 consistent synonymous and non-synonymous mutations that occurred in both passage lines and were maintained through to the end of passaging (Figure 2.4). There were four synonymous mutations (nucleotide positions 282, 737, 745, and 1153) and three non-synonymous mutations (940, 1066, 1081) in ORF1. Within the CP gene, there was only one synonymous mutation. However, the synonymous mutation at 3461 on CP changed a codon from isoleucine to threonine in the nested ORF4 gene. In the RT ORF5, which is important for aphid transmission, two non-synonymous mutations (nucleotide positions 3706 and 5007) and two synonymous mutations (nucleotide positions 3830 and 4232) occurred. In addition, there were also four mutations (nucleotide positions 5284, 5285, 5288 and 5627) in the 3’-UTR region. The $d_{\text{d}N}/d_{\text{d}S}$ rate was 0.4 for the ORF1 and 2 genes, 0.12 for the CP ORF3 gene, 0.37 for the ORF4 gene, and 0.55 for the RT ORF5 gene. These $d_{\text{d}N}/d_{\text{d}S}$ values are consistent with a population under weak selection.

In serial transmission on soybeans by *N. bakeri*, there were 23 mutations total (Figure 2.4). In contrast to peas, many of these mutations (11) changed amino acids. Two non-synonymous mutations (nucleotide positions 309, 940 and 941) occurred in ORF1, three (nucleotide positions 1452, 1796, and 2757) occurred in ORF2, two (nucleotide positions 3090 and 3392) in ORF4 (these mutations had no effect on the amino acid sequence of the coat protein), and four (nucleotide positions 3706, 4810, 4837, and 5050) on the RT ORF5. There were three synonymous mutations (nucleotide positions 282, 810 and 1153) in ORF1. Three mutations were found (nucleotide positions 9, 64 and 126) in the 5’-UTR, and four mutations were also found (nucleotide positions 5627, 5775, 5776 and 5782) in the 3’-UTR. Unexpectedly, the mutation at nucleotide position 309 changed the reading frame from a tyrosine codon to a stop codon. The $d_{\text{d}N}/d_{\text{d}S}$ rate was 1.25 for the ORF1 and 2 genes, 0.34 for the CP ORF3 gene, 1.13 for the ORF4 gene, and 0.82 for the RT ORF5 gene. The higher $d_{\text{d}N}/d_{\text{d}S}$ values for ORF1 and 2, ORF4 and ORF5 are consistent with a population under positive selection. In the clover control line, no non-synonymous
mutations were observed, and only 2 synonymous mutations (nucleotide position 2027 and 5095) were found after 4 serial transmissions (data not shown).

For the back inoculation of passage 6 and 8 in pea transmission lines, the sequences of the mutation regions were checked. Most of consistent mutations that were identified in serial transmissions described above reverted to the same as sequences in the original clover, including three non-synonymous mutations (nucleotide position 1066, 1081, and 5007). There are only 3 synonymous mutations (nucleotide position 282, 3830 and 4232) preserved when the viral populations were transmitted from pea or soybean back into the clover.

**DISCUSSION**

In the serial transmission experiments, SbDV-MD6 was transmitted to peas with high efficiency (67%) from the beginning of the passaging when using 10 *A. pisum* per pea, and maintained a high level of transmission efficiency thereafter. When three aphids were used for the inoculation, the most dramatic improvement of transmission efficiency was observed after the third transmission, and the improvement in transmission efficiency was much easier to distinguish from the beginning to the end of the passage experiment (Table 2.1). It would appear that the efficiency of transmission to pea was so good that the only way to see the subtle improvement with continued passage on the same host is to limit the inoculum load presented at each passage. Quantitative real-time RT-PCR suggested that viral titers in pea passage lines were increasing by the second passage and significantly increasing by P7 (Figure 2.5). Symptom severity on infected peas increased with serial transmissions, as the later passages of infected peas became significantly shorter and smaller than the initial passage (Figure 2.3A). The increases in SbDV titer, disease symptom severity, and transmission efficiency all suggest that SbDV-MD6 was adapting to the peas.

In contrast to the pea serial transmission lines, the transmission efficiency on soybeans was initially low, and it decreased with serial transmission on soybean, despite the fact that a significantly higher number of aphids were used in soybean inoculations (Table 2.1). The competency of the vector could be a factor in the poor transmission efficiency. However, *N. bakeri* typically transmitted SbDV from infected clover to healthy clover with an average of 66% efficiency (data not shown) and transmitted from infected clover
to soybean with 33-53% efficiency following the first or second passage to soybean (Table 2.1). Fluctuations in transmission efficiency may result from inconsistent aphid feeding behavior on the non-preferred host (soybean). While soybean is not the preferred host, *N. bakeri* will feed on soybean to acquire and transmit the virus, and were observed to survive and feed on soybean for the duration of the 5-day inoculation access period. In addition, if the aphid vector competency was the sole factor limiting transmission efficiency, one would expect that the transmission efficiency would remain relatively constant. The qRT-PCR data indicated that SbDV-MD6 was adapting to soybeans as a host during the serial transmission process. Viral titers in soybeans improved dramatically after the third passage, and overall the SbDV-MD6 population in soybeans demonstrated an approximately 6 fold increase in viral concentration based on real time RT-PCR (Figure 2.5). An alternative explanation for the loss of transmission efficiency in soybean passage lines is the possibility that host adaptive mutations in viral populations passaged on soybean had deleterious effects for the aphid vector transmission. Aphid transmission of luteoviruses requires very specific interactions between aphids and viruses (Gray and Gildow, 2003). Although it would seem likely that transmission efficiency was not necessarily related to the host adaptation, it is not out of the realm of possibility that host adaptive mutations could have a deleterious effect on a separate biological process, aphid transmission.

Sequence analysis of the SbDV-MD6 genomes recovered from the pea and soybean host serial passages may suggest possible mechanisms for the observed increases in virus titer and the loss of virus transmission in soybean. The non-synonymous mutations that change amino acids would be expected to affect biological functions. In pea passages, three non-synonymous mutations were located in ORF1. This protein is directly involved in viral replication and is likely to interact with host proteins. An additional non-synonymous mutation was found in ORF4, which has been identified as the movement protein, another likely source of host directed selection. Two more non-synonymous mutations were found in the RTP ORF5, which has been suggested to contribute to tissue specificity within the plant host (Peter et al., 2009). In addition, it is important to remember that SbDV-MD6 may also be undergoing selection for transmission by *A. pisum* as the viral population is serially transmitted. There were four additional mutations
in the 3’-UTR of pea-passaged SbDV-MD6. The 3’-UTR is related to replication initiation, but this portion of the genome is rather variable amongst SbDV isolates.

In soybean passage lines, the SbDV-MD6 $d_\text{N}/d_\text{S}$ values were significantly higher than the $d_\text{N}/d_\text{S}$ values observed in pea passage lines. In soybean, positive selection was indicated for replication proteins (ORF1 and 2), the movement protein (ORF4), and the readthrough protein (ORF5). The coat protein was not under positive selection in soybean. In pea, none of the viral genes exhibited $d_\text{N}/d_\text{S}$ values indicating strong selection. This suggests that the viral populations making the host shift from clover to soybean encountered greater selection pressures than the SbDV-MD6 populations making the shift from clover to pea. The more dramatic improvements in viral titer over the course of serial passage on soybean would support this hypothesis. The sites where amino acids changed in SbDV-MD6 soybean passages are mainly located on replication and RT coding regions. The mutations in the replication related proteins (ORF1 and ORF2) and the 5’ UTR (important replication control sequence) could easily be related to host selection pressures, but it is harder to imagine the function of the three mutations in the C-terminal of the RTP.

Previous research suggests that RTP is related to vector transmission (Terauchi et al., 2003), and it is certainly possible that the RTP mutations are related to selection for N. bakeri transmission, but all evidence indicates that transmission efficiency by N. bakeri is actually decreasing in soybean serial transmissions. There may be additional functions for the RTP, such as host tissue specificity and intercellular transport (Brault et al., 2000a; Bruyere et al., 1997; Chay et al., 1996a; Mutterer et al., 1999; Peter et al., 2009), and it’s interesting to note that SbDV-MD6 accumulated non-synonymous mutations in the RTP serially passaged on peas. When these pea-adapted virus populations were back inoculated into clover again, most of mutations (including all of the non-synonymous mutations) that occurred in soybean reverted to the original clover sequence, further suggesting that these mutations were related to host adaptation. It is also interesting that one of the non-synonymous changes in soybean passage lines introduces a stop codon in ORF1, a critical protein for viral replication. However, all of the sequencing was performed directly on RT-PCR products, so it would be impossible to determine if the mutations were not present in a minority of the population without a more detailed
cloning analysis. In addition, an internal methionine codon is available for the initiation of translation, just downstream from the introduced stop codon. The significant improvement in viral titers suggested that at least a functional level of ORF1 protein products must be available to the viral population.

On the surface, the loss of aphid transmissibility in SbDV-MD6 soybean serial transmissions does not make sense. Certainly any viral population making such an adjustment in a field setting would be rapidly out competed by neighboring transmissible populations, regardless of replication efficiency within the host. However, it is important to remember that the format of the serial transmissions done here is heavily biased towards generating viral populations that are strongly adapted to the host. Although luteoviruses only infect host phloem cells, the viral populations are exposed to host related selection pressures in every infected cell, from the very beginning to the very end of the passage experiment. In contrast, the aphid vector itself constitutes a temporal selection pressure that is never actually associated with a replicating viral population. Under these conditions, if there is a mutation that is beneficial to the virus in the new host regime but deleterious to aphid transmission, this mutation would become dominant in the population even if the advantage in the host was minimal and the cost to transmissibility drastic.

These experiments might lead to speculation that soybeans are not really a viable alternative host for SbDV-MD6. A host that selects for a non-transmissible population would certainly seem to be an evolutionary dead end. However, that point of view would be flawed because of the host selection biased limitations of the experimental design. The conditions used here and in many other experimental systems do not necessarily reflect the reality for viruses in the field. In the field, particularly on perennial pasture hosts such as clover, aphids are a near constant presence. As such, the selection pressures for maintaining aphid transmissibility would almost certainly be stronger, and have a greater influence on the eventual makeup of the viral population. The role of near-continuous aphid transmission as a selection pressure on clover isolates of SbDV to adapt to new aphid vectors species or to new plant hosts has yet to be determined.
Acknowledgements

The authors wish to acknowledge the contributions of William Sackett for aphid rearing and plant handling.
Figure 2.1. Experimental design for the serial transmission of SbDV-MD6 in pea or soybean plants. The original infected clover (P0) was used as the source plant. Infected plants in the first passage (P1) were pooled to serve as the source for the next transmission. Each passage line was continued through eight passages (P8), or until transmission failed.
Figure 2.2. Sequencing strategy for determination of full length consensus sequences of SbDV-MD6. PCR fragments (thin lines) were generated using the primers (named above PCR fragments. Two additional primers (1090R, 4336F) were used to determine internal sequences of larger PCR fragments.
Figure 2.3. Comparison of healthy and SbDV-MD6 infected pea and soybean plants. (A) SbDV-MD6 infected showed increased dwarfing and stunted growth from passage 1 to passage 8 when compared to healthy control plants at 25 days post-inoculation. (B) SbDV-MD6 infected soybeans are yellowing compared with healthy soybeans. (C) SbDV-MD6 infected soybeans showed yellowing and leaf elongation, but no dwarfing at 40 d.p.i. (D) Statistical comparison of height and fresh weight of infected peas indicates significant differences between passages 1 and 9.
Figure 2.4. Locations of mutations that occurred on pea and soybean during serial transmissions. A schematic diagram of the host adapted SbDV-MD6 genomes with genes labeled are shown. Panel A represents all mutations observed in SbDV-MD6 from pea (hexagons above genome) and from soybeans (stars below genome). Panel B represents non-synonymous mutations observed in SbDV-MD6 from pea and from soybeans with amino acid changes shown on the second line.
Figure 2.5 Real time RT-PCR virus quantification of SbDV-MD6 during serial transmissions in pea-passage (A) and soybean-passage (B) lines at 30 days p.i.. Statistical significance levels (determined by one-way ANOVA, P<0.05) indicated by asterisks.

A

**SbDV Quantification of Pea Passages**

![Graph showing SbDV quantification in pea passages](image)

B

**SbDV Quantification of Soybean Passages**

![Graph showing SbDV quantification in soybean passages](image)
Table 2.1 The transmission efficiency of SbDV-MD6 on pea and soybean lines. The percentage is determined by infected plants of total plants in each experiment.

<table>
<thead>
<tr>
<th>SbDV-MD6</th>
<th>Passage</th>
<th>Host</th>
<th>Vector no./plant</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pea line 1</td>
<td>10 A.pisum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pea line 2</td>
<td>3 A.pisum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soybean line 1</td>
<td>&gt;30 N.bakeri</td>
<td>33%</td>
<td>7%</td>
<td>7%</td>
<td>33%</td>
<td>20%</td>
<td>7%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soybean line 2</td>
<td>&gt;30 N.bakeri</td>
<td>53%</td>
<td>46%</td>
<td>14%</td>
<td>7%</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

*The source plant is infected clover which was collected in the field; b Lower transmission efficiency attributed to use of older scening source plant that was less than optimal for aphid feeding and virus acquisition. c No data * Significant difference from passage 1 base on Chi-Squared Test, all passages were compared with first passage, p<0.01. For soybean passage, because there are not enough positive samples, no valid statistics could be done.
Table 2.2. The transmission efficiency of back-inoculating clover with SbDV-MD6 from pea using *A. pisum* and *N. bakeri*, and soybean passages using *N. bakeri* only.

<table>
<thead>
<tr>
<th>SbDV-MD6</th>
<th>Passagea</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea line 1</td>
<td>20 <em>N. bakeri</em></td>
<td>4/6</td>
<td>2/6</td>
<td>4/6</td>
<td>2/6</td>
<td>0/6</td>
<td>1/6</td>
<td>1/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Pea line 2</td>
<td>20 <em>A. pisum</em></td>
<td>4/6</td>
<td>-</td>
<td>-</td>
<td>1/6</td>
<td>0/6</td>
<td>0/6</td>
<td>2/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Pea line 2</td>
<td>20 <em>N. bakeri</em></td>
<td>5/6</td>
<td>-</td>
<td>-</td>
<td>2/6</td>
<td>1/6</td>
<td>-</td>
<td>2/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Soybean line 1</td>
<td>30 <em>N. bakeri</em></td>
<td>3/10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0/10</td>
<td>-</td>
</tr>
<tr>
<td>Soybean Line 2</td>
<td>30 <em>N. bakeri</em></td>
<td>2/10</td>
<td>-</td>
<td>-</td>
<td>1/10</td>
<td>0/10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Infected source plants used for aphid acquisition of SbDV-MD6 correspond to infected plants shown in Table 1. For soybean lines, only infected plants from the first and last passages were tried.

b No data
CHAPTER 3

Cell regulation of interaction between Soybean Dwarf Virus and aphid Vectors

Abstract

It is known that luteoviruses have a very specific interaction with aphid vectors. Previous studies suggested that there may be a trade-off effect between the viral host adaptation and aphid transmission when Soybean Dwarf Virus (SbDV) was transmitted into a soybean hosts by aphid vectors. When a clover strain of SbDV was sequentially transmitted through several soybeans, aphid transmission was eventually lost. To explain why SbDV could not be sequentially transmitted by certain aphid vectors in soybean, multiple SbDV isolates originating from clover and soybean sources were tested in transmission assays for their ability to be acquired into the aphid gut lumen and hemocoel and to be effectively transmitted. Virus titers in the different aphid vectors were examined during the courses of transmission assays by the real time RT-PCR. Results indicated that all aphids are able to acquire and retain SbDV into the hemocoel regardless of ability to then transmit the virus. Thus, SbDV mutations of the structural proteins selected by replication and/or movement in soybean did not inhibit SbDV transport into the aphid hemocoel and no gut-barrier to SbDV transmission was detected. This observation suggests that the most probable selective site in the aphid preventing transmission of soybean-adapted SbDV is at the accessory salivary gland. We hypothesized that selection pressure on the SbDV replication in soybean is probably leading to reduced virus capsid read-through protein recognition by aphid receptors in the accessory salivary gland resulting in reduced transmission efficiency.

Introduction

Soybean Dwarf Virus (SbDV) is a member of the Luteoviridae family, found in multiple locations around the world, including Japan (Tamada et al, 1969) and the United States (Damsteegt and Hewings, 1987). Like other luteoviruses, SbDV has specific vector relationships (Gray and Gildow, 2003), and these relationships appear to have played a role in the evolution and diversification of the virus (Terauchi et al., 2003). SbDV is exclusively transmitted by aphid vectors in a persistent, non-propagative manner, one of
the most crucial and most conserved features of luteoviruses. It is phloem-limited and occurs in low concentration in the plants. SbDV isolates have been found in many different plant hosts such as white clover, subterranean clover, broad bean, pea, and lentils (*Lens culinaris* Medik). However, it usually causes an economically important disease only in soybean crops. There are several distinct isolates based on symptomatology in soybeans, aphid vector specificity, and molecular makeup. The yellowing strains, SbDV-YS and SbDV-YP, cause severe interveinal chlorosis, rugosity and thickening of leaves in soybeans. The dwarfing strains, SbDV-DS and SbDV-DP, cause stunting with shortened internodes and brittle curled leaves. The YS and DS strains are transmitted specifically by the aphid *Aulacorthum solani*, while YP and DP strains are transmitted by both *Acyrthosiphon pisum* and *Nearctaphis bakeri*.

Luteoviruses have a very specific interaction with aphid vectors. The selective specificity of the luteovirus transmission can occur at a minimum of three cellular sites, including the gut cell membrane, and the cell membrane and basal lamina of the accessory salivary gland (ASG). These specific recognition sites are likely determined by multiple protein domains on the virus capsid and multiple cell surface receptors of aphid vectors (Gray and Gildow, 2003).

Luteovirus particle ingestion into the alimentary canal of aphids begins with the penetration of aphid stylet into virus-infected phloem sieve elements or companion cells. The first barrier encountered by a luteovirus after entering the aphid is the midgut or hindgut. Generally, the aphid gut does not play a critical role in virus/vector specificity. Most luteoviruses can penetrate the gut barrier of most common aphid vectors, entering into the hemolymph by the receptor mediated endocytotic/exocytotic process (Gray and Banerjee, 1999). Studies showed that vector *Myzus persicae* could acquire both Potato Leafroll Virus (PLRV) and SbDV, but through different cells of the midgut and hindgut, respectively (Garret et al., 1993; Gildow et al., 2000). There are exceptions where the gut plays a role in determining virus/vector specificity. One study indicated that Cereal Yellow Dwarf Virus-RPV could not penetrate the gut barrier of non-vector *Metopolophium dirhodum* (Gildow, 1993). These observations suggested that virus recognition at the gut is a prerequisite of virus acquisition, and different luteovirus-recognition receptors located in different regions of the gut determine the selective
specificity (Garret et al., 1993; Gildow, 1993, 1999).

Luteoviruses in the hemocoel cannot be transmitted until they contact the ASG, where two barriers have been identified: the basal lamina that consists of a complex extracellular matrix surrounding ASG cells, and the cell membrane (basal plasmalemma) of the ASG. For example, the penetration of Barley Yellow Dwarf Virus isolate-MAV (BYDV-MAV) through the basal lamina was blocked when fab fragments of the monoclonal anti-BYDV-MAV immunoglobulin G were injected into the vector aphid, *Sitobion avenae*. It suggested that virus capsid recognition and interaction with basal lamina surrounding the ASG might be important for vector specificity (Gildow and Gray, 1993). Study of SbDV-Va20, another SbDV isolate in the United States, indicated that a major barrier for SbDV transmission occurred on ASG cells, and that the vector selective specificity was recognized at both basal lamina and basal plasmalemma independently (Gildow et al., 2000). Another evidence is the electron microscopy observation of CYDV-RPV in the non-vector aphid *S. avenae*. The virus particles accumulate at the basal plasmalemma of the ASG, but never penetrate into the cytoplasm of ASG cells, thus never become transmissible (Gildow and Rochow, 1980). All these observations suggest a selective specificity system in luteovirus-aphids interaction at the ASG, where the accessory salivary basal lamina acts as a preliminary barrier filter, following by the cell plasmalemma as a second selective barrier.

Indigenous SbDV was found commonly in clover in the United States, but rarely caused disease on soybean (Damsteegt et al., 1999). It was suggested that SbDV outbreaks were limited because of the lack of aphids colonizing soybean before 2000 (Damsteegt et al., 2005). Since 2000, an aphid (*Aphis glycines*) capable of colonizing soybeans was introduced into the United States, and there are reports of SbDV infection in soybean crops in Wisconsin and Illinois (Harrison et al., 2005; Phibbs et al., 2004), Soybean aphids and SbDV isolates transmitted by soybean aphids were also discovered in the eastern US (Schneider et al., 2011). However, SbDV infections in fields were still limited, and only occurred on the very edges of the fields. There are two probable hypotheses explaining the lack of widespread epidemics of SbDV in soybean crops in the eastern US. One is that the clover isolate of SbDV has not yet adapted to the new soybean host. The other probable explanation is that SbDV could not be efficiently transmitted by
the newly introduced soybean aphids, which are the only aphids capable of colonizing and reproducing on soybeans. In the previous study (Chapter 2), we found that virus titers of SbDV-MD6 isolate increased in the soybean plants when the virus was serially passaged, but aphid transmission ability by *Nearctaphis bakeri* was eventually lost. Our results suggested that there was a tradeoff between the host adaptation and aphid transmission. The mutations accumulated during serial transmissions were beneficial for virus replication and/or movement in soybeans, but had negative effects on aphid transmission. The objective of this study was to explain the reason SbDV-MD6 could not be sequentially transmitted by certain aphid vectors on soybeans, and to better identify the cellular regulation mechanism of SbDV transmission.

**METHODS AND MATERIALS**

**Plants, viruses, and aphid vector**

Four SbDV isolates were used in this study. SbDV-MD6 and –MD16 (both YP strain isolates) were obtained from different locations in Maryland. Japan D (a DS strain isolate) and Japan Y (a YS strain isolate) isolates were obtained from the Hokaido prefect of Japan in 1993. All isolates maintained in white clover (*Trifolium repens*). Puget pea (*Pisum sativum* cv. Puget), soybeans (*Glycine max* cv. Williams), and white clover seedlings used for host serial transmissions were maintained the same as described above (Chapter 2). Red clovers (*Trifolium pratense* L.) were used as non-host feeding for aphid vectors. In the aphid transmission experiments, *A. pisum* (pea aphid), *N. bakeri* (clover aphid) and *A. glycines* (soybean aphid) were used as the vectors for serial transmission and maintained as described above (Chapter 2).

**Virus acquisition and retention assays**

For the serial passages, aphids acquired viruses by feeding on detached leaves of infected white clover as source for 24 hrs. Then, the aphids were transferred to healthy pea or soybean seedlings for 5 days. At the same time, three healthy seedlings of each plant species were fed on by healthy aphids as a negative control. After 25 days post-inoculation, the percentage of infected plants was determined by ELISA according to manufacturer directions (Agdia, Elkhart, IN). Each passage line was continued through
four passages, or until transmission failed. SbDV-MD6 was transmitted by both aphids in the white clover, as a positive control for transmission.

To test for virus acquisition by aphids from SbDV-infected plants of each passage, 100 *N. bakeri* were put on each of 12 SbDV-infected soybean seedlings for a 24hr acquisition feeding at 20°C. Infected soybeans 30 days post-inoculation were used as virus sources at each passage. Approximately 100 *A. pismum* were acquisition fed in parallel on 10 SbDV-MD6 infected pea plants. After the 24hr acquisition feeding, two sets of 25 aphids on each infected plant were collected immediately, flash frozen by liquid nitrogen and stored at -80°C for RNA extraction. The remaining aphids of each species were then transferred to healthy red clover for 2 days. The SbDV-MD6 does not infect or replicate in red clover and during this feeding time the aphid gut lumen is likely cleared of ingested virus. Following the 2-day feeding on red clover, 25 aphids of each plant were flash frozen, and stored at -80°C for RNA extraction and analysis for SbDV retention in the aphid hemocoel.

**Comparison of SbDV isolates for acquisition efficiency**

Soybean plants infected with different isolates of SbDV (MD6, MD16, Japan D and Japan Y) were used as source plants for aphid acquisition and retention test. Each isolate was passaged at least 3 times in soybeans prior to use. SbDV infection of each soybean source plant was confirmed by real time PCR. For virus acquisition, about 75 *A. glycines* or *A. pismum* were put on each of 3 or 4 infected soybeans for a 48hr acquisition feeding. Immediately following the acquisition feeding, 15 aphids were removed at random and flash-frozen for RNA extraction and real time RT-PCR analysis for viral RNA. The remaining aphids were transferred to healthy red clover plants for 2 days to allow SbDV to clear the alimentary canal lumen. Then 15 aphids were collected at random from each plant and flash frozen with the same procedure as described previously. Individual samples consisted of RNA extracted from the combined total of 15 aphids fed on each source plant. Preliminary studies indicated that multiple aphid samples were required for consistently positive detection of SbDV in samples. Each treatment consisted of aphid samples from either 3 (*A. pismum*) or 4 (*A. glycines*) infected soybeans. Results of real time PCR analyses expressed as the log10 values of target copies per ng total RNA.
(indicated by RQ log10 values, and the formula is described in Chapter 2) are values averaged from all positive reactions for each treatment.

**Total RNA extraction and real time RT-PCR**

In each passage, the same number of aphids was collected in the tube and flash frozen by liquid nitrogen, and stored at -80°C for RNA extraction. The aphid total RNA extraction employed the same protocol as Wallis *et al.*, 2007. The final RNA pellet was dried by vacuum and resuspended in 40µl distilled water. SbDV RNA was amplified using first strand cDNA synthesis as described above (Chapter 2). The real time RT-PCR followed the same protocol as previous reported (Chapter 2). Similarly, to determine the effectiveness of quantification, a fragment of SbDV-MD6 was cloned into a plasmid with a T7 RNA polymerase promoter. This plasmid was linearized and used to make *in vitro* transcripts, which served both as positive controls for qRT-PCR and as a directly quantifiable RNA template to establish standard curves.

**Results**

To examine how serial passages on soybean and pea affect aphid transmission efficiency, SbDV-MD6 was initially transmitted from clover to pea or soybean by *A. pisum* and *N. bakeri*, respectively, and then repeatedly transmitted to the same host plant by the same aphid species for 4 sequential transmissions. The transmission efficiency of each aphid species was then determined following each transmission or passage (Table 3.1). Transmission efficiency of SbDV-MD6 for *A. pisum* fed sequentially on peas for passages 1-4, was 30, 40, 60, and 50%, respectively. This confirmed the previous result (Chapter 2) that ability of *A. pisum* to transmit SbDV-MD6 to peas increased in efficiency in passages following the first adaptive transmission from clover to peas. Previous studies (Chapter 2) have indicated that SbDV titer increased in peas and soybeans with each succeeding transmission during serial passages. Therefore, one might expect transmission efficiency to increase on both host plant species in later passages, as was demonstrated for the pea treatment. However, transmission efficiency of *N. bakeri* fed sequentially on soybeans transmitted SbDV in passages 1-4 at 25, 16, 8, and 0%, respectively. Therefore, the results suggested the same phenomena observed in Chapter 2.
that SbDV transmission by *N. bakeri* was reduced with each subsequent passage in soybeans until transmission ability was lost after the third passage.

To examine the affect of sequential serial passages on abilities of *A. pisum* and *N. bakeri* to acquire and retain SbDV, virus titers in aphids allow to acquire viruses from each of the four passages were analyzed by real time PCR. To acquire virus, aphids were allowed a 24hr acquisition feeding on infected plants from each passage, and then two sets of 25 aphids were analyzed by real time PCR. A cohort of 25 aphids from each passage was allowed to feed 2 days on red clover (non-host) to clear virons in the alimentary canal and then tested similarly. Results of PCR analyses from two separate experiments (Table 3.2) indicated that both *A. pisum* and *N. bakeri* effectively acquired SbDV into the gut during a 24hr feeding on pea or soybean and retained the virus in the hemocoel following a 48hr feeding on red clover to clear the gut lumen content. In *A. pisum* on pea, SbDV concentrations (indicated by RQ Log10 values) acquired into the hemocoel were consistently about 7.00 for viruses acquired following passages 1 to 3. In *N. bakeri* on soybeans, SbDV accumulation in the hemocoel at passage 1 was lower than that recorded for passage 3. This observation indicated that SbDV was acquired into the hemocoel of *N. bakeri* at a concentration higher than observed for aphids acquiring virus at passage 1. This was to be expected because SbDV was shown to increase in concentration in soybeans through sequential passages over time (chapter 2). Aphids of passage 3, however, transmitted the virus at a much lower efficiency (8%) compared to aphids of passages 1 (25%) and passage 2 (16%). After passage 3, *N. bakeri* were unable to transmit the SbDV even though PCR data indicated relatively high concentrations of SbDV in soybeans and in aphid hemocoel.

To compare acquisition and retention of diverse SbDV isolates from different geographical and plant host species in potential aphid vectors, different isolates of SbDV (MD-6, MD-16, Japan-Y, and Japan-D) were established in soybeans through 3 serial passages using *N. bakeri*. Then two different aphid vectors, *A. pisum* and *A. glycines*, were acquisition fed 24hr on the infected soybeans, and 15 aphids removed for real time PCR to detect the virus titers acquired into the aphids gut lumen and tissues. The remaining aphids were removed from the infected soybean source and given a 48hr feeding on non-host red clover to remove residual virus from the gut lumen and 15 aphids
were tested by real-time PCR for retention in the hemocoel. Japan D isolate was used as a negative control because it could not be transmitted by either *A. glycines* or *A. pisum*. The results (Table 3.3) showed that Japan D could be acquired by *A. glycines* and *A. pisum* but was not detected after being fed on the non-host red clover. Japan Y is also not transmitted by either *A. pisum* or *A. glycines*, but viruses were acquired and detected in *A. glycines*, but not in *A. pisum*. This would suggest that *A. glycines* can acquire SbDV-Y but transmission is likely prohibited by an accessory salivary gland barrier, whereas in *A. pisum* a gut barrier might prevent SbDV acquisition. The Ct values in both vectors are high, approaching the negative Ct value of 40 (RQ Log10 = 3.9), indicating that it is not likely present and both aphid species acquire SbDV-Y inefficiently. For SbDV-MD6 and SbDV-MD16, both *A. glycines* and *A. pisum* acquired and retained both viruses in the hemocoel. Following a 48 hr feeding on non-hosts compared to concentration values following the initial 24 hr acquisition feeding on infected plants suggest that SbDV uptake from the gut lumen and transport into the hemocoel is inefficient. However, both species do acquire SbDV-MD6 and SbDV-MD16 into the hemocoel and are genetically competent to transmit the virus as indicated in transmission studies (Table 3.3).

**Discussion**

In previous studies (Chapter 2), we reported that the MD6 clover strain of SbDV showed increased fitness and increased aphid transmission efficiency when adapting to a new host species, pea. Following serial passages on soybean, SbDV-MD6 also adapted readily to soybean by improved replication and/or movement. Selection for host adaptation to soybean, however, created tradeoff effects decreasing host-to-host transmissibility by aphid vectors. Therefore, we investigated the mechanisms behind this tradeoff phenomenon, and the factors that affect the interaction between SbDV and aphid vectors.

Transmission of luteoviruses requires multiple virus-recognition, transcellular transport, and endocytic/exocytic steps in the vector to achieve circulative transmission. Luteovirus transmission can be prevented or blocked by mutations in virus structural proteins disrupting recognition at any steps of the sequence. If the aphid does acquire virions into the gut lumen while feeding, there may be barriers to acquisition by endocytosis into the aphid gut cells or subsequent release into the hemocoel. Last, if virus
does circulate into the hemocoel and reach the accessory salivary gland, virions may be inhibited from penetrating the ASG, basal lamina or basal plasmalemma, thus, preventing transmission. When SbDV-MD6 was passaged on soybeans, mutations were observed on ORF1, ORF2, and ORF 5 (Chapter 2). ORF1 and 2 are replication related genes, and ORF5 is a readthrough extension of coat protein that has been proven to be related to aphid transmission (Brault et al., 2007). The most likely factors that affect aphid transmission were the mutations accumulated on ORF5. However, it is possible that the mutations on ORF1 and ORF2 affected virus distribution, movement, or recognition in such a way that acquisition of the virions from the infected plant became more difficult.

In an experiment designed to determine if the lack of transmission was related to virus acquisition and/or retention by potential aphid vectors, four isolates of SbDV were tested for transmission by A. glycines and A. pisum. The U.S. isolates MD6 and MD16 were both transmissible by both aphid species, and Japan D and Y isolates were not transmissible by either aphid species. The results indicate that all four isolates were ingested by both aphid species, regardless of whether or not the isolates were transmissible by that particular aphid species. Difference among isolates of SbDV did not affect the acquisition of virus from soybean by aphids. From the table 3.2, transmissible virus/vector combinations resulted in higher titer in aphids than the non-transmissible virus/vector combinations, despite the fact that all infected plants had similar titer of virus concentration (data not shown).

After 24hrs acquisition feed, the aphid were removed to a non-host plant that were suitable feeding sources for the aphids. Aphids were allowed to feed on non-host plants for 48hrs to clear the gut of virions acquired from infected soybeans. At this point, the only SbDV left in the aphids should be virus that has passed the gut barrier into the hemocoel. Following the non-host feeding, the transmissible isolates MD6 and MD16 were retained in both aphid species, and non-transmissible isolates Japan D and Y were not retained in the aphids, except for the Japan Y isolate in A. glycines. This suggests that in the aphid A. pisum, the gut was the barrier for Japan D and Y into the hemocoel. In A. glycines, Japan D was not able to enter the hemocoel, and Japan Y isolate was able to enter the hemocoel only at low efficiency. MD6 and MD16 were able to enter the hemocoel of both aphid vectors with variable efficiency. MD6 isolate was efficiently
transported into hemocoel by \textit{A. pisum} but not efficiently by \textit{A. glycines}. MD16 was more efficiently taken up by \textit{A. glycines} than \textit{A. pisum}, despite the fact that both MD6 and MD16 are efficiently transmitted by \textit{A. pisum} and poorly transmitted by \textit{A. glycines} (Damsteegt et al., 2011). Based on these results, the acquisition of Maryland isolates of SbDV by aphids was not a major problem for aphid transmission. The gut was the barrier for Japan D and Y into the hemocoel of \textit{A. pisum}. Although \textit{A. glycines} is a poor vector for MD-6 and -16, aphids still retained viruses successfully. Our observations reported here indicates that other selective sites must exist to limit \textit{A. glycines} transmission of soybean adapted MD-6 and -16. The most probable site is at the ASG which is known for specific interaction between aphid and virus in many systems. As with other luteovirus/aphid vector relationships, SbDV appears to have multiple levels of vector specificity to overcome.

To investigate if the viral mutations accumulated in the serial passages affected virus transport into the aphid hemocoel and the ASG, serial transmissions of MD6 were carried out on pea and soybean by two efficient vectors, \textit{A. pisum} and \textit{N. bakeri}. The previous results indicating that transmission on pea remained the same or improved to high levels for both vector species suggested that mutations that occurred when MD6 shifted host from clover to pea have little effect on aphid transmission. However, the eventual loss of transmission ability via \textit{N. bakeri} on soybean suggests that mutations accumulated in soybean host somehow interrupt the specific interaction between aphid and viruses.

It is surprising that SbDV populations undergoing selection for new hosts would suffer tradeoff effects with regards to viral transmission. However, the compact nature of RNA virus genomes often results in multifunctional viral proteins in which a minor mutation increasing selection for one functional trait could result in interference with a second functional trait of the same protein. There are numerous examples of multifunctional viral proteins, including the RTP of luteoviruses (Peter et al., 2009; Terauchi et al., 2003; VandenHeuvel et al., 1997). Although this work does not address the direct effects of SbDV-MD6 mutations acquired during serial passage in soybeans, the fact that the same mutations occurred in three independent passage experiments resulting in the same loss of transmission by multiple vector competent aphid species strongly suggests that the mutations are somehow both advantageous in soybean and interfering with the
transmission of MD6 into the ASG.
Table 3.1 Transmission efficiency of SbDV-MD6 on pea and soybean. The percentage is determined by infected plants divided by total plants in each experiment.

<table>
<thead>
<tr>
<th>Host Plant</th>
<th>Aphid vector</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pea</td>
<td><em>A. pisum</em></td>
<td>30% (3/10)</td>
<td>40% (4/10)</td>
<td>60% (6/10)</td>
<td>50% (3/6)</td>
</tr>
<tr>
<td>Soybean</td>
<td><em>N. bakeri</em></td>
<td>25% (3/12)</td>
<td>17% (2/12)</td>
<td>8% (1/12)</td>
<td>0 (0/15)</td>
</tr>
</tbody>
</table>

Source plant: SbDV-MD6 infected clover
Table 3.2 Relative concentration of SbDV-MD6 in the aphid through serial passages. The 
$C_t$ values in real time PCR are indicated by RQ Log10 values.

<table>
<thead>
<tr>
<th>Passages</th>
<th>Vector</th>
<th>$A. pisum$</th>
<th>$N. bakeri$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Line 1</td>
<td>Line 2</td>
</tr>
<tr>
<td>P0</td>
<td>Acquisition feeding</td>
<td>- $^{a}$</td>
<td>ND $^{b}$</td>
</tr>
<tr>
<td></td>
<td>Non-host feeding</td>
<td>-</td>
<td>ND $^{b}$</td>
</tr>
<tr>
<td>P1</td>
<td>Acquisition feeding</td>
<td>6.67+/-.03</td>
<td>6.72+/-.14</td>
</tr>
<tr>
<td></td>
<td>Non-host feeding</td>
<td>-</td>
<td>6.88+/-.12</td>
</tr>
<tr>
<td>P2</td>
<td>Acquisition feeding</td>
<td>6.05+/-.05</td>
<td>7.47+/-.14</td>
</tr>
<tr>
<td></td>
<td>Non-host feeding</td>
<td>6.28+/-.04</td>
<td>6.51+/-.08</td>
</tr>
<tr>
<td>P3</td>
<td>Acquisition feeding</td>
<td>6.82+/-.00</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Non-host feeding</td>
<td>-</td>
<td>7.04+/-.04</td>
</tr>
</tbody>
</table>

$^{a}$ Negative reaction  
$^{b}$ None done  
$^{c}$ Mean RQ Log10 values for all positive results with standard deviations. In cases where only a single sample tested positive the standard deviation is listed as 0.0.
Table 3.3 Relative concentration of soybean Dwarf Virus (SbDV) in the aphid vectors, *A. glycines* and *A. pisum*, following a 24hr acquisition on soybeans infected with the North American SbDV isolates MD6 and MD16, and the Japanese isolates D and Y feeding and a 48hr non-host feeding, as indicated by RQ Log10 values.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Vector</th>
<th>Acquisition efficiency</th>
<th>Average RQ Log10 in aphid&lt;sup&gt;b&lt;/sup&gt;</th>
<th>24hr acquisition feeding</th>
<th>48hr non-host feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD6</td>
<td><em>A. glycines</em></td>
<td>1/4</td>
<td>1/4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.22 +/- 0.0</td>
<td>4.45 +/- 0.0</td>
</tr>
<tr>
<td></td>
<td><em>A. pisum</em></td>
<td>3/3</td>
<td>2/3</td>
<td>7.31 +/- 0.15</td>
<td>6.65 +/- 0.17</td>
</tr>
<tr>
<td>MD16</td>
<td><em>A. glycines</em></td>
<td>2/3</td>
<td>2/3</td>
<td>5.73 +/- 0.02</td>
<td>6.07 +/- 0.16</td>
</tr>
<tr>
<td></td>
<td><em>A. pisum</em></td>
<td>3/3</td>
<td>2/3</td>
<td>7.09 +/- 0.10</td>
<td>5.26 +/- 0.08</td>
</tr>
<tr>
<td>Japan D</td>
<td><em>A. glycines</em></td>
<td>3/4</td>
<td>0/4</td>
<td>6.41 +/- 0.14</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td><em>A. pisum</em></td>
<td>3/3</td>
<td>0/3</td>
<td>5.97 +/- 0.23</td>
<td>-</td>
</tr>
<tr>
<td>Japan Y</td>
<td><em>A. glycines</em></td>
<td>4/4</td>
<td>3/4</td>
<td>5.63 +/- 0.38</td>
<td>4.86 +/- 0.29</td>
</tr>
<tr>
<td></td>
<td><em>A. pisum</em></td>
<td>3/3</td>
<td>0/3</td>
<td>4.52 +/- 0.12</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Acquisition and retention values represented by two fractions. The first fraction represents the number of times the aphid acquired the virus from feeding on infected host tissue/total number of experimental repetitions. The second fraction represents the number of times the aphid retained the virus after feeding on non-infected non-host tissue/total number of experimental repetitions.

<sup>b</sup> Mean Ct values for all positive results with standard deviations. In cases where only a single sample tested positive the standard deviation is listed as 0.0. The PCR reaction limits of detection are about 5fg for both D and Y assays (typical 5fg = 4.33 (RQ Log10)). Negative reactions indicated by (-)

For each sample, RNA was extracted from 15 aphids fed on SbDV infected soybean from the third passage.
CHAPTER 4
Determining the genetic diversity of Soybean Dwarf Virus in multiple host plants

Abstract
Characteristically, RNA viruses have genetically diverse populations within a single host. Important biological characteristics may be related to the levels of diversity, influencing host adaptability, host specificity, and host range. Changes in virus population diversity may be associated with changes in host species. The level of genetic diversity for these viruses is related to host-virus interactions and understanding these interactions may facilitate the prediction and prevention of emerging viral diseases. The levels of population diversity of Soybean Dwarf Viruses (SbDV) in three different plant hosts: pea (Pisum sativum), soybean (Glycine max), and clover (Trifolium repens) were examined in this study. The levels of diversity over the course of serial passage were also examined. Results indicated that the diversity of SbDV populations initially increased when SbDV was first transferred to a new host species, but remained relatively constant over subsequent serial passages in the new host. Sequence analysis of SbDV populations revealed biases for particular types of substitutions and identified regions of the genome susceptible to mutations among different hosts. Most mutations are transient and/or deleterious although they were generated frequently within a single plant host.

Introduction
Population genetic diversity is the essential component that allows a species to adapt to an ever-changing environment. The error-prone replication, large population, and rapid replication times associated with RNA viruses lead to genetically diverse populations even within a single host (Roossinck, 1997). It has been thought that unlike most animal viruses, most plant viruses need to be generalists for survival (Garcia-Arenal et al., 2003). Viral populations are complex and dynamic when the level of genetic diversity in a population reacts to changes in selection pressures (Lech et al, 1996). Maintaining a high level of genetic diversity would be an advantage for a virus to access a new environment, such as a different, distantly related host species (Holmes, 2009). Most comparative studies of viral evolution have utilized consensus sequences that represent something of
an average of the entire viral population in single host, but more detailed studies of evolutionary dynamics relying on genetic diversity would require more detailed gene resolution (Holmes, 2009). From limited analyses of intra-host diversity data, some studies indicate that the diversity level of plant viruses correlates to the size of their reported host range (Schneider and Roossinck, 2000). Meanwhile in different hosts, plant viruses also have different ability to maintain diverse populations (Schneider and Roossinck, 2001). For instance, in Wheat Streak Mosaic Virus, the diversity level did not change significantly when passaged in different cereal species (Hall et al, 2001). In contrast, members of the family Flexiviridae have been reported to maintain high diversity levels and a strong constrained pressure on coding sequences (Teycheney et al, 2005; Chare and Holmes, 2004; Shi et al, 2004).

Genetic diversity of some viruses is entirely related to genomic survival associated with bottleneck size related to virus transmission, host infection, or host survival. Transmission bottlenecks have been documented during a process of inter-host transmission, particularly as mediated by aphid vectors (Ali et al, 2006). Phylogenetic analysis of coat proteins of different plant viruses indicated that vector-borne viruses were subject to significantly stronger purifying selection than non-vector-borne viruses (Chare and Holmes, 2004). Similar population bottlenecks have also been observed during the mosquito stage in the vector-borne RNA viruses that infect animals (Smith et al, 2008). However, there are some indirect studies of viral population which suggest that transmission bottlenecks in nature may not be as extensive as is often thought (Holmes, 2009). Detailed studies of Dengue Virus intra-host genetic diversity suggested that transmission bottlenecks are not especially severe, where the defective RNA viruses are still able to spread in the population with complementation by co-infection of hosts with functional viruses (Aaskov et al, 2006). Similarly, multicomponent viruses requiring mixed multiparticle infections to form a fully functional unit could be considered to select against a transmission bottleneck in the case of some plant RNA viruses (Manrubia, et al., 2005).

Little is known about the evolutionary process and genetic diversity of populations in luteoviruses, which are highly vector-specific and host-tissue-specific (Gildow, 1999). Transmission bottlenecks have been considered a key factor to reduce effective
population size that limits genetic diversity of luteovirus populations, especially as related to virus particle structural proteins associated with transmission. The luteovirus Soybean Dwarf virus (SbDV) is a single-stranded, positive-sense RNA virus. ORF-1 and -2 encode essential elements for replication, and ORF-3, -4 and -5 encode proteins involved in structure, movement, and vector transmission (Mayo and Miller, 1999).

Our previous studies (Chapter 3) indicated that there is a trade-off effect between the SbDV fitness in soybean and aphid vector transmissions when SbDV was transmitted from clover to soybean sequentially in the greenhouse. However, there are few data about the effects of transmission bottlenecks on intra-host genetic diversity. In this study, using various plant host species as the changing environment and sequential transmissions on the same new host species as the constant environment, the genetic diversity levels of viral populations in different hosts and serial passages were examined to provide an insight into vector effects on the luteovirus genome, particularly the replicase-related regions. Identifying vector effects affecting the diversity level of viral populations is an important step to the understanding and management of vectored pathogens of new/emerging plant diseases.

Materials and Methods

Plants and viruses
Infections with SbDV were initiated with a SbDV population from wild clover plants. Plant hosts include soybean and pea. Two independent passage experiments were done by aphid transmission as previously described in Chapter Two. Briefly, SbDV was transmitted from naturally infected white clover (Trifolium repens) (passage 0) to soybean (Glycine max), and pea (Pisum sativum) by the aphids N. bakeri and A. pisum, respectively. Infected soybeans or peas (passage 1) were used as the virus source for the next transmission, continuing through 6 or 8 passages. Two individual infected plants were randomly selected from the first passage in both host species, passage 6 in soybean and passage 8 in pea.

Extraction of total RNA and cDNA synthesis
Total RNA from original, naturally infected clover was extracted, and thirty days post-
inoculation total RNA was extracted from systemically infected leaves of infected pea leaves of passage 1 and passage 8, and infected soybean leaves of passage 1 and passage 6 as previously described (Chapter 2). Total RNA extraction was used as a template for reverse transcription (RT) with Superscript reverse transcriptase as prescribed by the manufacturer (Invitrogen). Thermal cycling reactions were carried out for 20 cycles (94°C denaturation for 30s, 52°C annealing for 1 min, 72°C extension for 1 min), and included a polymerase with proofreading capability (Pfu; Invitrogen). There were three PCR products generated covering ORF1 and ORF2 region of SbDV-MD6. The primers used in the PCR assay were 1UF (5’-GAC TAT GGG TTT GAC ATG CAG-3’) and 1090UR (5’-GTT TGA ATC CCC GTT TTC T-3’), 900UF (5’-GCA ACC ATC AAC CGA TAT GCG-3’) and 2126UR (5’-GTT TGA ATC CCC GTT TTC T-3’), and 1235UF (5’-GTT TGA ATC CCC GTT TTC T-3’) and 2959UR (5’-GAG TGC TTC TAT TTT GAA AGT ATT GG-3’).

Cloning and sequencing of viral populations
The PCR products of viral RNAs from individual plants from each treatment were cloned separately and treated as unique populations. The amplified products generated from the viral RNAs were cloned into the vector pCR2.1-TOPO (Invitrogen) and sequenced with both directions. The viral clones were sequenced at the Nucleic Acid Facilities (The Pennsylvania State University). Eleven to 20 clones were sequenced from each individual infected plant. The alignment of all the cloning sequences were done by using Bioedit (Ibis Therapeutics, Carlsbad, CA) and checked manually. Changes between the sequences of the viral population clones and the consensus sequence of the source population were recorded as mutations. In cases where multiple mutations occurred in close proximity in the same clone, each mutated base was considered a unique mutation. The mutation frequency was calculated as the total number of mutations observed in all clones for a given viral population divided by the total number of bases sequenced for the population.

Statistical analysis
Comparisons between viral populations in different hosts were tested for statistical significance using the ANOVA (analysis of variance) test from the statistical package Minitab 15 (Minitab Inc., State College, PA) to determine least significant differences.
Comparison of mutation free zones was done between species using sequencing alignment.

**Results**

To identify the extent and structure of intra-host viral genetic diversity of SbDV in different plant hosts with serial passaging, we sequenced the clones from 9 samples. SbDV-MD6 from infected clover described in Chapter 3 was used as the inoculation source. Young pea and soybean plants were inoculated by aphid vector *A. pisum* and *N. bakeri*, respectively, and passaged for 8 or 6 times as previously described. Total RNA was extracted from 2 infected plants for each passage, and the viral populations were cloned separately. This RNA was used as a template for the reverse transcription, and the resulting cDNA was amplified with virus isolate-specific primers. The amplified products included 3 fragments covering approximately 2.5 kb in length, which included the replication-related genes (ORF1 and ORF2) and some flanking sequences. They were cloned separately and 11 to 20 clones were analyzed for each fragment from each virus population. The mutation frequencies were very similar and no statistical difference between passage lines for both hosts, so mutation frequencies within the passages were pooled.

In theory, each clone represented a unique viral RNA, and comparing the sequences of the viral clones to the consensus sequence provided a snapshot of the genetic diversity generated within a given viral population. Using these clones as a representative sample of viral populations, mutation frequency and the percentage of mutated viral clones were used as indicators of population diversity. Control reactions were done using *in vitro* transcripts as the template RNA to estimate the level of variability introduced by transcription, RT, and thermal cycling. There are 4 of 22 control clones (18%) containing a single mutation, giving the background level of experimental mutation frequency at 0.02%, significantly lower than levels observed in the viral populations.

**Mutation frequency**

Mutation frequency is the number of bases that differ from the consensus sequence divided by the total number of bases sequenced. Two plants were sampled for each plant
treatment. Cloning and sequencing two RT-PCR sets for each treatment would detect potential differences in levels of error introduced in independent RT-PCRs. There were no significant differences between the sampled plants of any given passage, so the data from clones for each passage were pooled. Comparisons between treatments were done using ANOVA tests to determine least significant differences.

The replication-related genes (ORF1 and ORF2) were used to exam the genetic diversity of SbDV population in a single infected plant. The level of intra-host genetic diversity increased from 0.08% on average in clover to 0.11% in peas and 0.12% in soybeans after reaching equilibrium. After SbDV was passaged in the new host for several times, the level of diversity decreased slightly to 0.10% in both pea and soybean passages. There were no significant changes over the courses of passaging in the same host species. The only significantly different mutation frequency observed in the plant occurred in passage 1 soybeans where the mutation frequency reached the 0.12%.

**Mutation distribution**

An examination of the locations of the mutations suggests that mutations were randomly distributed but not evenly distributed (Figure 4.2). The observed mutations in the SbDV populations were distributed throughout the sequenced region, with a bias for the ORF1 (from nucleotide position 145 to 12301) region over the ORF2 region (from nucleotide position 1221 to 2828). There were some areas where many mutations were observed, in particular the area between nucleotides 257 and 1046 of ORF1 gene, but no mutation hot spots were observed. In addition, there was a region from 1942 to 2238 where mutations occurred on the ORF2 region, and there were two mutation free regions on ORF2.

**Type of mutations**

All of the observed mutations in SbDV populations were substitutions except one insertion in the pea passage 1. Close examination of the specific changes indicates a bias for transitions, in particular A-to-G and U-to-C transitions. There was a strong bias for A-to-G transitions in both pea and soybean passages (61 of 116 substitutions in pea and 58 out of 130 substitution in soybean, Table 3). The SbDV populations in clover demonstrated a slight preference for U-to-C (13 substitutions) over A-to-G (6
There are more synonymous mutations observed than non-synonymous in pea passages but no bias was observed in the clover and soybean populations (Table 4.2). Occasionally, bases close to one another mutated. In these cases, each individual mutated base was counted as a mutation, even though the mutations may have arisen from a single mutational event. However, if these mutations are grouped and counted as a single mutation, it does not affect the statistical comparisons of mutation frequencies significantly.

Discussion
Previous studies on SbDV passages in different plant hosts suggest that some important mutations on SbDV replication-related genes may be associated with changes in biological functions in the plant host. The mutation observed in both pea and soybean passaged SbDV-MD6 included mutation in ORF1 and ORF2. However, exact functions of these mutations remain unknown. Based on the results of chapter 2 and 3, we know that a tradeoff effect between viral replication and aphid transmission occurs when SbDV infecting clover moved to a soybean host. This study is a controlled comparison of changes in SbDV-MD6 viral population diversity during passages in new hosts, focusing on the ORF1 and ORF2 regions.

The levels of SbDV population diversity increased significantly when SbDV moved from clover to soybeans at passage 1, however, subsequent passages have no significant difference. This suggests that the SbDV is capable of generating diversity when shifting to a new host, and other factors limit the eventual accumulation of variation in whole plants. Results indicated that SbDV-MD6 was capable of adapting to new hosts very rapidly. It is interesting to note that the greatest increase of SbDV-MD6 virus diversity occurred under the strong selection pressure during the shift to the soybean host, as compared to peas. The diversity of SbDV-MD6 maintained statistically similar levels for all other populations except for passage 1 in soybeans. This is different from other viruses, where different levels of diversity are observed on different hosts (Schneider and Roossinck, 2000). However, it is the first observation of population diversity in luteoviruses. The fact that luteovirus are limited to phloem may contribute to similarities
in mutation frequencies between hosts, as the phloem environment may be similar for virus replication from host to host. In addition, the three hosts used in this study were all related legumes.

The level of intra-host diversity we report for SbDV (mean = 0.10%) is in the normal range as that recently observed in intra-host studies of other plant and animal RNA viruses (Schneider et al., 2000; Murcia et al., 2010). Therefore, SbDV appears to exhibit mutational dynamics broadly similar to those observed in some rapidly evolving RNA viruses, and is as expected given the intrinsically error-prone nature of replication with RNA-dependent RNA polymerase. Our results are compatible with the notion that the majority of intra-host mutations in SbDV are deleterious and removed by purifying selection when the virus is maintained in one host species. In addition, the few mutations dominant in the final passages especially in soybeans suggests that positive selections do occur when SbDV is moved to new hosts by aphid vectors. This suggests that the bottleneck imposed by the aphid is not substantial in this persistent transmission manner, which may play an important role in selection pressure on viruses.

Transitions are the most common mutations. In this study, the transitions from A-G and U-C are the most common substitutions. In both pea and soybean, A-G is more common than U-C, but in clover, U-C is the most common mutations observation. This may indicate that the environments in different hosts are not exactly the same. The A-G and U-C transition bias is caused by base pairing between the guanidine and uridine (Schneider and Roossinck, 2000), and has been noted in other RNA viruses (Schneider and Roossinck, 2001). The occurrence of A-G or U-C transitions is related to whether U-G base pairing occurs during positive or negative strand synthesis. The preference for U-C transition in clover may suggest that more mutation occur during the negative strand synthesis in that host.

There were three regions on SbDV-MD6 replicase–related genes where mutations were not observed. One region (nucleotide 658 to 846) is in ORF1, and two large regions are on ORF2. Overall, ORF1 had more mutations than ORF2. This may suggest that the mutation free regions on ORF2 are conserved and important for virus replication. Mutations likely do occur in these mutation free regions, but viral RNAs with mutations in these regions are selected out of population. It is also important to note that the cloned
viral populations are being selected for at the RNA level. There are more non-mutated copies of genes to produce functional replicases. Therefore, the presence of regions where mutations are not recovered indicates the presence of selection for RNA sequence. SbDV has regions with higher mutation rates, which suggests that the mutation free regions are not occurring by chance. The observed mutations are not restricting the infection of SbDV. If so, deleterious mutations would be rapidly selected out of the population.

It is clear that selection plays an important role, because mutations are biased for synonymous changes in pea host (Table 4.1), and they are not evenly distributed (Figure 4.1). We also assume that bottlenecks associated with persistent aphid transmission and long-distant movements in plant host may limit the diversity, because the observed mutation frequency in whole plants is low, and even in the most densely mutated regions it does not approach the theoretical mutation frequency.

Theoretically, the ability to maintain genetic diversity in viral populations should enhance chances for adaptation to new selective regimes. Alternatively, if high diversity in the viral population resulted in fitness losses with Muller’s Rachet theory (Escarmis et al., 2009), the forces of selection would rapidly eliminate viruses that surpass viable limits of population diversity. This study has important evolutionary implications for the intra-host genetic structure of viral populations since it is the first study of a phloem-limited luteovirus. It has been suggested that clover strains of SbDV have a chance of expanding into a new niche and thus pose a threat of emerging as new crop disease of soybeans. However, the bottleneck imposed by persistent aphid transmission may be the major factor to limit the spread of SbDV in the nature.

It is difficult to identify a mechanism that would select a specific level of diversity for host-virus relationship (Schneider and Roosinck, 2000). One possible scenario could be that the viral replicase may cause different error rates due to host components associated with viral replication process. Any environmental factors, such as concentrations of available nucleotides, pH, and other soluble components, may affect the fidelity of viral replicase complex. Alternatively, these viruses may be capable of generating equivalent levels of diversity in different hosts, but some selection pressure specific to a particular host, and/or a bottleneck will act as a cap, limiting the accumulation of diversity. For the
case of SbDV-MD6, all hosts and all passages were statistically indistinguishable in terms of genetic diversity, with the exception of the initial passage in soybean. Previous data (chapter 2) suggests that this initial passage on soybeans is the point at which SbDV-MD6 is undergoing the most significant changes (based on number of non-synonymous changes and changes in viral titers, or fitness). It would be premature to say that the increased level of SbDV-MD6 is directly associated with the stringent selection for adaptation to a new host, but the observation is interesting and warrants further study.
Table 4.1 Genetic variation in SbDV-MD6 populations in different host species and passages.

<table>
<thead>
<tr>
<th>Host and passage</th>
<th>% mutated clones</th>
<th>Total mutations/bases sequenced</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clover</td>
<td>60% (6/10)</td>
<td>25/29,590</td>
<td>0.08%</td>
</tr>
<tr>
<td>Pea and passage 1</td>
<td>55% (11/20)</td>
<td>66/59,180</td>
<td>0.11%</td>
</tr>
<tr>
<td>Pea and passage 8</td>
<td>40% (8/20)</td>
<td>64/59,180</td>
<td>0.10%</td>
</tr>
<tr>
<td>Soybean and passage 1</td>
<td>56% (10/18)</td>
<td>64/53,262</td>
<td>0.12%*</td>
</tr>
<tr>
<td>Soybean and passage 6</td>
<td>44% (8/18)</td>
<td>53/53,262</td>
<td>0.10%</td>
</tr>
</tbody>
</table>

Substitutions are counted for determining mutation frequency. Least significant differences were determined using the ANOVA test (*P <0.05).
Table 4.2 Types of mutations observed in SbDV-MD6 populations in different host species and passages.

<table>
<thead>
<tr>
<th>Host and passage</th>
<th>Translated</th>
<th></th>
<th>Non translated</th>
<th></th>
<th>Total substitutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Syn</td>
<td>Non-syn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clover</td>
<td></td>
<td>15</td>
<td>7</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>Pea and passage 1</td>
<td></td>
<td>43</td>
<td>18</td>
<td>5</td>
<td>66</td>
</tr>
<tr>
<td>Pea and passage 8</td>
<td></td>
<td>48</td>
<td>13</td>
<td>3</td>
<td>64</td>
</tr>
<tr>
<td>Soybean and passage 1</td>
<td></td>
<td>34</td>
<td>26</td>
<td>3</td>
<td>63</td>
</tr>
<tr>
<td>Soybean and passage 6</td>
<td></td>
<td>20</td>
<td>32</td>
<td>1</td>
<td>53</td>
</tr>
</tbody>
</table>

Syn – synonymous mutation
<table>
<thead>
<tr>
<th>Host</th>
<th>Original bases</th>
<th>No. of substitutions at mutated base</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G</td>
<td>A</td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>Clover</td>
<td>G</td>
<td>-</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>6</td>
<td>-</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Soybean</td>
<td>G</td>
<td>-</td>
<td>6</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>58</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Pea</td>
<td>G</td>
<td>-</td>
<td>16</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>61</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.1 Genetic diversity in new hosts increase, followed by reaching equilibrium. Individual passages (P1, P6, P8) are represented by ovals, with the percentage of mutated clones per passage represented as a fraction in parentheses, and the overall diversity level represented by a percentage. Two independent passage lines were established for each host, and the total data combined to determine genetic diversity levels for each host during the initial and final passage.

*P<0.05
Figure 4.2 Distribution of accumulated mutations observed in clones derived from SbDV-MD6 populations. The region sequenced extends from base 1 to base 2959 covering both ORF1 and ORF2 regions. Mutation-prone areas are indicated by dark blocks, and mutation free zones are white regions on the map.
CHAPTER 5

Conclusions and Future Directions

Plant viruses cause many important diseases in agriculture and are responsible for great losses in crop yield and quality worldwide. Soybean Dwarf Virus (SbDV) is an important plant pathogen infecting soybean and causing high economic losses throughout the Asia (Tamada et al., 1969). In the United States, SbDV primarily infects clovers and was previously not known to cause diseases in soybean, presumably because of the absence of competent aphid vectors. Recently, an imported soybean aphid, *Aphis glycines*, has been detected in more than 20 states (Wang et al., 2006). Although *A. glycines* is not an efficient vector of SbDV, the SbDV is able to be transmitted by *A. glycines* occasionally (Damsteegt et al., 2011). It is important to understand the characteristics of SbDV, since its biological properties involving evolution and transmission will not only increase overall knowledge of virus evolution and viral transmission specificity, but will also provide potential strategies to evaluate the risk of SbDV epidemics, as well as to monitor and control the disease.

In this study, mutations were identified in passaged clover isolates of SbDV that might be associated with host shifts and/or vector transmission. The clover isolate SbDV-MD6 was serially transmitted from clover to pea or soybean by two aphid vectors, *Acrythosipon pisum*, and *Nearctaphis bakeri*. Sequence analysis SbDV-MD6 from both pea and soybean passages identified 6 consistent non-synonymous consistent mutations in pea, compared to 11 mutations in soybean transmitted by *N. bakeri*. The \( d_{S}/d_{S} \) analysis indicated that SbDV was under strong selective pressures in soybean, but not in pea. Significantly increasing virus titers with each sequential transmission supports this analysis. However, aphid transmission efficiency on soybean decreased with each passage until the virus was no longer aphid transmissible. These results indicated that the clover strain of SbDV-MD6 adapts readily to soybean by improved replication and/or movement, but selection for host adaptation creates trade-off factors decreasing host-to-host transmissibility by aphid vectors.

To further study the reason for the trade-off between the host adaptation and aphid transmission and the cellular regulation mechanism of SbDV transmission, diverse isolates of SbDV, including Japan Y, Japan D, SbDV-MD16 and SbDV-MD6, were used
in transmission assays utilizing *A. psium* and *A. glycines*. Although virus titers increased in soybean host plants, serial transmission by *A. glycines* ceased after only one or two passages in soybean. Results indicate that all aphids were able to acquire the virus into the gut lumen while feeding. After two days feeding on a non-host plant, however, some non-vectors of Japan Y and D tested negative by PCR for virus indicating these viruses were prevented from entering the aphid hemocoel by a gut barrier. Both *A. pisum* and *A. glycines* retained MD-6 and -16 in their hemocoel following the feeding on the non-host species. However, *A. glycines* still failed to transmit MD-6 and -16 from soybean. This suggests that the most probable site blocking transmission occurred at the accessory salivary gland, which is known for specific interaction between aphid and virus in many luteovirus systems. We hypothesized that clover isolates of SbDV occurring in N. America when adapting to the soybean host undergo selective mutations enhancing SbDV replication and/or movement in soybean which are detrimental to aphid transmission. In future research, electron microscopy will be used in an attempt to confirm the locations of SbDV-MD6 in the aphid and identify the transmission barriers. This information is needed for a better understanding of the cellular regulation of virus/vector specificity, particularly regarding the fate of SbDV particles that can successfully pass the gut barrier.

In addition, the examination of SbDV-MD6 population diversity in different host species indicated that the diversity of SbDV populations increased slightly when at the beginning of shifting to a new host, then remained relatively constant over the serial passages. The initial passage in soybean, where data indicates the most significant adaptation is occurring, is the only environment where SbDV-MD6 generated levels of genetic diversity that were significantly higher than the levels of diversity found in other hosts or later in the soybean passage experiments. The explanation for this is not clear, but needs to be investigated further. It provides evidence that SbDV has a chance of expanding into a new niche and thus poses a threat of emerging as a new crop pathogen. However, the bottleneck imposed by persistent aphid transmission may be the major limitation to SbDV spread in nature.

Taken together, this information will allow us to better assess the risk of indigenous SbDV infecting and spreading in annual soybean crops while utilizing wild perennial
legumes as virus overwinter survival sites and reservoirs for aphid vectors, and provide insight into the SbDV evolution and the potential risk of disease epidemics caused by SbDV.
References


Brault, V., Mutterer, J., Scheidecker, D., Simonis, M.T., Herrbach, E., Richards, K.,


Aphid transmission and systemic plant infection determinants of barley yellow dwarf luteovirus-PAV are contained in the coat protein readthrough domain and 17-kDa protein, respectively. Virology 219, 57-65.


Phytopathology 83, 1293-1302.


Yang, X.L., Thannhauser, T.W., Burrows, M., Cox-Foster, D., Gildow, F.E., Gray, S.M., 2008. Coupling genetics and proteomics to identify aphid proteins associated with vector-
specific transmission of polerovirus (Luteoviridae). J. Virol. 82, 291-299.
Curriculum Vitae

Bin Tian

EDUCATION EXPERIENCE

Ph.D., Plant Pathology (2007- August 2012) The Pennsylvania State University, University Park, PA
M.S., Plant Science (2004-2007) Institute of Botany, Chinese Academy of Sciences, Beijing, China
B. S., Biology (2000-2004) Beijing Forestry University, Beijing, China

SELECTED PUBLICATIONS


PRESENTATIONS

“Understanding the mechanism and evolution of vector transmission, vector specificity and host specificity for Soybean Dwarf Virus”, Department of Plant Pathology, Ohio State University, 2012

“Soybean Dwarf Virus: Adaptation to New Hosts and Vectors”, Department of Plant Pathology, Penn State University, 2011


“Adaptation of Soybean Dwarf Virus to New Host Species”, 11th International Plant Virus Epidemiology Symposium, Ithaca, NY, 2010

AWARDS/FUNDING

Graduate Student Travel Award, 2011, College of Agricultural Science, Penn State.

APS Student Travel Award, 2011: The evolutionary genetics of emerging plant viruses. The Albert Paulus Student Travel Award, The American Phytopathological Society.

Travel Assistance Award, 2010: Lab training for RT-qPCR design and massively parallel sequencing pathogen detection. Department of Plant Pathology, Penn State.