

ROLE OF VIRUS GENES IN SEED AND APHID TRANSMISSION AND DEVELOPMENT
OF A VIRUS-INDUCED GENE SILENCING SYSTEM TO STUDY SEED DEVELOPMENT
IN SOYBEAN

BY

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DISSERTATION

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ABSTRACT

Soybean mosaic virus (SMV) and *Tobacco streak virus* (TSV) are two important seed transmitted virus that infect soybean. SMV is a member of the family *Potyviridae* genus *Potyvirus*, and is transmitted by seed and aphids in soybean. The symptoms of SMV infection on soybean typically include mosaic and curling of leaves, reduced pod set and seed coat mottling. Although, the virus genes responsible for transmission by aphids have been studied extensively there has not been much research in understanding virus genes responsible for seed transmission. In this study I investigated the role of SMV genes in seed and aphid transmission, by designing recombinants between SMV isolates, SMV 413 (highly seed and aphid transmitted) and SMV G2 (lacking seed or aphid transmission). The SMV genes encoding the proteins P1, helper component protease (HC-Pro), and coat protein (CP) were the major determinants of seed transmission. The single amino acid mutation of G12 to D in the DAG motif of the CP and the amino acid Q264 to P in the C-terminus of the CP affected seed transmission of SMV. The aphid transmission study validated previous research and demonstrated that the amino acid motif DAG in the CP region and HC-Pro were important in aphid transmission. The severity of foliar symptoms was influenced by HC-Pro and seed coat mottling was determined by a single amino acid Q264 in the C-terminus of the CP. The seed transmitted virus TSV (genus *Ilarvirus* and family *Bromoviridae*) was utilized for construction of a DNA-based virus induced gene silencing (VIGS) vector for investigating genes expressed in the soybean seed. VIGS are reverse genetics tools comprised of virus vectors with a partial plant transcript insertion that can silence expression of the plant gene. TSV has a tripartite genome, and causes early symptoms such as leaf puckering and necrosis that are often followed by symptom recovery possibly induced by

virus RNA silencing. Multicloning sites were inserted into all the three RNAs of TSV to facilitate the insertion of plant gene fragments for the development of a VIGS vector. TSV RNA2-based VIGS vector was the most stable and the integrity of plant gene inserts up to the size of 175 nucleotides were maintained in the vector. The small RNA (21-24 nucleotide size) deep sequencing of TSV-infected soybean plants with symptom recovery revealed that virus derived small interfering (vsi)RNA originated from hot spots within the TSV genome as indicated by the number of mapped sequences. The vsiRNA hot spots were not correlated to the presence of RNA secondary structure in the TSV genome and exhibited a bias for the sense strand. Most hot spots were located in the TSV RNA3 especially in the movement protein coding region. To further understand the effects of virus infection on micro (mi)RNA accumulation and function in plants showing symptom recovery, small RNAs and mRNAs were sequenced from three soybean cultivars either mock-inoculated or infected with TSV or *Bean pod mottle virus* (BPMV). BPMV (genus *Comovirus* and family *Comoviridae*) also causes severe symptoms initially followed by symptom recovery in soybean as observed in TSV. The soybean miRNA, gma-miRNA159a, that targets transcripts of a glutathione S-transferase (GST) and a MYB family transcription factor, and gma-miR166 that targets a HD-Zip transcription factor were down-regulated in virus-infected soybean exhibiting symptom recovery as indicated by the number of mapped sequences to the reported gma-miRNA transcript sequence. The GST, MYB factor and HD-Zip factor proteins may all be involved in programmed cell death that is associated with plant disease resistance. The mRNA-Seq analysis revealed that the accumulation of mRNAs encoding pathogenesis-related proteins, components of the salicylic acid mediated defense response and plant RNA virus infection associated proteins were up-regulated in virus-infected plants with symptom recovery. The transcripts for GST and lactoylglutathione lyase

protein, involved in detoxification, were down-regulated. Changes in the accumulation of transcripts from other defense- and photosynthesis- related genes were cultivar dependent, as determined from the number of sequences that mapped to soybean transcripts.

To my daughter Nyasa

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CHAPTER 1

GENERAL INTRODUCTION: *SOYBEAN MOSAIC VIRUS* AND *TOBACCO STREAK*

VIRUS IN SOYBEAN

Soybean, *Glycine max* (L.) Merr., is an important oil seed, averaging approximately 40% protein and 20% oil by weight (Ash, 2010). It is the largest source of protein feed for animals and is the second largest source of vegetable oil used for human consumption. Soybean is the dominant oilseed in the US accounting for 90% of the US oilseed production. An area of 77.5 million acres was under soybean production in 2009 in the US making it the second most planted crop after corn. The US is the worlds largest producer and exporter of soybean (Ash, 2010).

SOYBEAN MOSAIC VIRUS (SMV)

Several viruses infect soybean, among which, SMV is one of the most damaging, infecting soybean worldwide. Soybean mosaic disease was first reported from Connecticut in 1915 (Clinton, 1915) and was described in 1921 (Gardner and Kendrick, 1921). The losses caused by SMV infection can be up to 35% depending on the incidence of infection and the stage at which the plants become infected. Early infection on soybean can result in reduced pod set, seed size and weight with low seed oil content and poor root nodulation (Hill, 1999). SMV infections can result in poor seed quality and cause seed coat mottling (Hobbs et al., 2003). High incidences of SMV have been reported late in the season from soybean fields in Iowa, although SMV is not prevalent in commercial soybean fields in Illinois (Hobbs et al., 2010.; Lu et al., 2010). SMV has

been divided into different strains, G1 through G7, depending on symptom development, from mosaic or necrosis to no symptoms, on a differential set of soybean lines (Cho and Goodman, 1979).

Transmission of SMV

SMV is aphid and seed transmitted in soybean (Domier et al., 2007). Seed transmission of SMV plays an important role in the epidemiology by spreading the disease temporally and over large distances (Mink, 1993). Seed transmission is the source of primary inoculum, since SMV rarely infects other hosts in the field (Hill, 1999). In SMV, seed transmission is dependent on the survival of virus in the embryo during seed maturation (Bowers and Goodman, 1991; Bowers Jr and Goodman, 1979). Successful seed transmission requires both virus movement into and replication within reproductive tissues (Johansen et al., 1994). Viruses can infect the embryo either indirectly or by infecting the megaspore or the pollen mother cells before embryo formation or directly by invading the embryo (Maule and Wang, 1999). *Pea seed-borne mosaic virus* (PSbMV, genus *Potyvirus*) infects embryos directly through symplastic connections between the maternal cells and the embryo (Robert et al., 2003). PSbMV gains access into the endosperm from the maternal tissue through plasmodesmal channels between the testa and endosperm cells. PSbMV can then invade the suspensor cells during the early seed development stage through transient vesicles present at the base of the suspensor in the micropylar region where the suspensor is anchored to the endosperm. The virus then enters the embryo from the suspensor through plasmodesmata (PD). Hence, the presence of the virus in the micropylar region at early seed development stage is necessary for seed transmission (Robert et al., 2003).

Spread of SMV from plant to plant is mainly by aphids. More than 32 species of aphids that do not colonize soybean transmit SMV in a nonpersistent manner (Hill, 1999). In the US, no colonizing aphids were reported on soybean until the discovery of the soybean aphid, *Aphis glycines*, in 2000, in Wisconsin and Illinois (Clark and Perry, 2002; Hartman et al., 2001). The soybean aphid, which overwinters on buckthorn (*Rhamnus* spp) and migrates to soybean plants in the spring and can transmit SMV very efficiently in the laboratory (Clark and Perry, 2002; Ragsdale et al., 2004). *A. glycines* can serve as a major vector of SMV in the field (Burrows et al., 2005), although other studies indicate that the role of *A. glycines* on spread of SMV is minimal (Pedersen et al., 2007).

SMV genome and gene functions

Taxonomically, SMV is from the genus *Potyvirus* and family *Potyviridae*, and has flexuous rod-shaped particles with a single stranded positive sense RNA of 9.5 kb (Adams et al., 2005). The genomic RNA of the virus is packaged by multiple copies of coat protein (CP) subunits. The genomic RNA has a polyadenylate tail at the 3' end and a viral genome-linked protein (VPg) covalently bound at the 5' end. The potyvirus genomes contain a single open reading frame (ORF) that is translated as a polyprotein that undergoes autocatalytic proteolysis to yield 9-10 mature protein products (Adams et al., 2005). The SMV gene products from the polyprotein are P1, helper component proteinase (HC-Pro), P3, PIPO, cylindrical inclusion (CI), 6K, VPg, nuclear inclusion a (NIa), nuclear inclusion b (NIb) and CP (Lopez-Moya and Garcia, 2008) (Fig. 1.1).

The P1 protein is the most variable protein among potyviruses and functions as a serine protease for self-cleavage from HC-Pro (Adams et al., 2005; Verchot et al., 1991). The P1 protein is also a silencing suppressor along with HC-Pro and has been suggested to play an important role in virus host range (Anandalakshmi et al., 1998; Rajamaki et al., 2005; Salvador et al., 2008; Valli et al., 2006). HC-Pro is a multifunctional protein in potyviruses that, in addition to other activities, interacts with CP for aphid transmission and functions as a cysteine proteinase (Atreya et al., 1990; Carrington et al., 1989; Ng and Falk, 2006; Oh and Carrington, 1989). P3 is a membrane-bound protein that interacts with the endoplasmic reticulum (ER) and Golgi apparatus in the cell and moves along actin microfilaments suggesting an involvement in virus replication and intracellular movement (Cui et al., 2010; Eaitanaste et al., 2007). PIPO (pretty interesting potyviral ORF), a recently discovered 25-kDa protein produced by a translational frame shift near the middle of the P3 cistron, forms a complex with the CI protein that can interact with the plasmodesmata facilitating cell-to-cell virus movement (Chung et al., 2008; Wei et al., 2010b; Wen and Hajimorad, 2010). The CI protein also functions as a RNA helicase involved in virus replication (Lain et al., 1990)

The 6K protein is another membrane associated protein that can form vesicles associated with the ER that may act as sites for virus replication or RNA translation (Cotton et al., 2009; Wei et al., 2010a). The VPg, as mentioned earlier, is bound to the 5'-termini of genomic RNAs and interacts with the translation initiation factor, eIF4E, and HC-Pro for virus RNA translation (Puustinen and Makinen, 2004; Roudet-Tavert et al., 2007). NIa protein is a serine protease that cleaves all the proteins from the polyprotein except HC-Pro and P1 (Carrington et al., 1993;

Hellmann et al., 1988). The NIb is the RNA-dependent RNA polymerase involved in virus replication (Domier et al., 1987). The goal of this study was to understand the influence of different SMV proteins and amino acids on seed and aphid transmission.

Role of HC-Pro and CP in potyviruses

HC-Pro and CP are multifunctional proteins that interact in multiple stages in the potyvirus infection cycle. In *Tobacco etch virus* (TEV), a potyvirus, cell-to-cell and long-distance movement are associated with CP and HC-Pro (Cronin et al., 1995). Multiple overlapping functional domains have been identified in the 457 amino acid long HC-Pro polypeptide (Fig. 1.2). Cell-to-cell movement of potyviruses, which is facilitated by increases in PD size exclusion limits, is controlled by 293 amino acids at the C-terminus of HC-Pro and core region of the CP (Rojas et al., 1997). Substitutions of conserved amino acids at positions 154 (R to D) and 198 (D to R) in the core region of the CP in TEV inhibited cell-to-cell movement in tobacco (Dolja et al., 1994). The CCCE amino acid motif in the central region of the HC-Pro was shown to be associated with systemic movement of TEV within plants (Cronin et al., 1995).

The central and C-terminal regions of potyviral HC-Pro have been implicated in suppression of posttranscriptional gene silencing (PTGS) (Kasschau and Carrington, 2001; Varrelmann et al., 2007). Single-amino-acid mutations in the highly conserved FRNK motif (Fig. 1.2) in HC-Pro of the potyvirus *Zucchini yellow mosaic virus* (ZYMV) reduced binding of HC-Pro to small interfering RNAs and micro RNAs (Shibolet et al., 2007). A single-amino acid substitution at position 134, from L to H, in the central domain of the HC-Pro significantly reduced the ability

of HC-Pro of *Plum pox virus*, a potyvirus, to suppress PTGS (González-Jara et al., 2005). The IGN motif in the central region of the HC-Pro (Fig. 1.2) is associated with genome amplification in TEV (Cronin et al., 1995), which likely results from suppression of PTGS. In PSbMV, HC-Pro was reported to be a major determinant of seed transmission (Johansen et al., 1996).

HC-Pro and CP are required for aphid transmission in potyviruses (Atreya et al., 1990; Ng and Falk, 2006). HC-Pro and the CP interact and bind to specific sites within aphid stylets for nonpersistent virus transmission. Aphid transmission of potyviruses involves sequence-specific interactions of two conserved amino acid motifs in HC-Pro and a single motif in CP. The N-terminal KITC/KLSC motif in the HC-Pro binds HC-Pro dimers to aphid stylets (Blanc et al., 1998; Seo et al., 2010). The second motif, PTK, which is located in the C-terminal region of HC-Pro, binds with the N-terminal DAG motif in CP (Blanc et al., 1997; Blanc et al., 1998; Peng et al., 1998) (Fig. 1.2; 1.3). This results in the transient binding of virus particles through HC-Pro to aphid stylets. The N-terminal region (amino acids 1 - ~100) of HC-Pro in TEV is exclusively involved in aphid transmission, and deletions of this region produce fully infectious viruses that are not transmitted by aphids (Dolja et al., 1993). Seed transmission and aphid transmission show similar trends, with SMV strains with low aphid transmission also exhibiting poor seed transmission (Domier et al., 2007). In this study, I examined the role of HC-Pro, CP and amino acid motifs in CP in seed and aphid transmission in SMV.

HC-Pro has been associated with symptom severity in potyviruses, SMV and ZYMV (Desbiez et al., 2010; Lim et al., 2007). HC-Pro of *Potato virus A*, a potyvirus, binds to VPg that interacts with eIF4E for a cap-independent translation, and in addition interacts directly with eIF(iso)4E,

another translation initiation factor, through a conserved C-terminal YINFLA motif (Ala-Poikela et al., 2011). The CP has been suggested to be part of the potyviral replication complex along with some host proteins in SMV (Seo et al., 2007).

TOBACCO STREAK VIRUS

Another destructive disease of soybean is caused by *Tobacco streak virus* (TSV), which can produce obvious acute symptoms, that later reduce in severity as plants recover from infection (Fulton, 1978; Xin and Ding, 2003). TSV can invade both reproductive and vegetative meristems in soybean resulting in high rates of seed transmission and bud-blight disease, respectively (Almeida et al., 2005; Demski et al., 1999; Ghanekar and Schwenk, 1974). The recovery phenomenon is associated with degradation of viral RNAs by PTGS (Baulcombe, 2005).

TSV was first reported on soybean from Brazil in 1955 (Costa et al., 1955). The initial symptoms of TSV infection on soybean include a yellow mosaic that later becomes systemic (Demski et al., 1999). In studies conducted in Wisconsin, TSV incidence on soybean was most severe during the R2 growth stage and symptom severity declined later during the season (Rabedeaux et al., 2005). Stunting often is one of the most pronounced symptoms of TSV infection on soybean. Other symptoms include leaf puckering and darkening along petioles and veins, with crooked stem tips. Severe symptoms include pod necrosis and bud blight that can lead to death in some plants (Almeida et al., 2005; Fagbenle and Ford, 1970; Ghanekar and Schwenk, 1974).

Transmission

TSV has been reported to be seed transmitted at rates of 2.6 to 30.6% among different soybean cultivars (Ghanekar and Schwenk, 1974). TSV has a wide host range infecting crop plants including cotton, dahlia, grape, peanut, potato, rose and tomato (Demski et al., 1999). TSV can also infect the dicotyledonous weed, *Ageratum houstonianum*, that can serve as a primary source of inoculum for TSV infection in the field (Greber et al., 1991). TSV is transmitted by thrips species *Frankliniella occidentalis*, *Microcephalothrips abdominalis* and *Thrips tabaci* in the field (Greber et al., 1991; Kaiser et al., 1982). Thrips transmission of TSV is associated with the presence of virus-infected pollen (Greber et al., 1991; Sdoodee and Teakle, 1987). Transmission is presumed to occur by virus present in pollen grains that infect the plant through wounds caused by thrips feeding (Sdoodee and Teakle, 1987)

TSV genome and gene functions

TSV belongs to the genus *Iilarvirus* and family *Bromoviridae* (Hull, 2002). Iilarviruses have quasi-isometric particles varying from spherical to bacilliform. TSV has a single-stranded (ss), positive-sense, tripartite RNA genome (Hull, 2002). RNA1 of TSV has 3,491 nucleotides and is monocistronic (Scott et al., 1998) (Fig. 1.4). RNA2 has 2926 nucleotides and codes for a protein (2a) of 800 amino acids and a second protein (2b) of 205 amino acids (Scott et al., 1998) (Fig. 1.4). RNA3 has 2,205 nucleotides, with a protein of 289 amino acids coded from 870 nucleotides on the 5' terminus-proximal coding region and a second protein of 237 amino acids from 714 nucleotides at the 3' terminus proximal coding region (Cornelissen et al., 1984). A subgenomic,

RNA4 is produced to express the 3' terminus proximal CP coding region of RNA3, which is needed for TSV infectivity and, by analogy to *Alfalfa mosaic virus*, may substitute for the poly (A)-binding protein in translation initiation (Krab et al., 2005; Vloten-Doting, 1975) (Fig. 1.4).

The protein encoded by RNA1, 1a, contains methyltransferase and helicase motifs. The larger ORF of RNA2 encodes 2a, the RNA-dependent RNA polymerase (RdRp). Both the 1a and 2a proteins are required for virus replication (Scott et al., 1998). The smaller ORF of RNA2 encodes the 2b protein that, by relation to the 2b protein of *Cucumber mosaic virus* (CMV), has been suggested to be involved in cell-to-cell movement of TSV within the host (Shi et al., 2003; Xin et al., 1998) and suppression of PTGS (Brigneti et al., 1998). The 5' terminus-proximal ORF of RNA3 codes for a movement protein (MP), and the 3 terminal proximal end encodes CP (Cornelissen et al., 1984).

VIGS system in soybean

Virus induced gene silencing (VIGS) reduces expression of a gene of interest that has been inserted into the viral genome either by replacement of a virus gene not essential for the infection and spread, or by addition of an extra gene fragment within the virus genome. In the plant cytoplasm, the modified virus containing a portion of the gene of interest can induce gene-specific silencing by triggering PTGS-mediated degradation of endogenous mRNA (Baulcombe, 1999; Voinnet, 2001). The goal of my experiments was to construct a TSV-based VIGS system for studying soybean seed development. VIGS is a powerful tool based on RNA and DNA viruses that has been used in many functional genomics studies in several plant systems. VIGS

has been used in identifying biotic and abiotic stress-related genes and understanding the role of specific genes in developmental biology and metabolic pathways (Senthil-Kumar et al., 2008). VIGS has been also used to simultaneously silence multiple unrelated genes by using gene fragments from multiple genes (Campbell and Huang, 2010; Peele et al., 2001; Turnage et al., 2002).

In soybean, a VIGS system has been developed and refined using the *Bean pod mottle virus* (BPMV), a bipartite comovirus, and has been used to study soybean genes responsible in defense, translation and cytoskeleton structure (Zhang and Ghabrial, 2006; Zhang et al., 2009). Although BPMV has been used for VIGS to identify gene and characterize genes in soybean, e.g., genes for resistance to soybean rust (Meyer et al., 2009) and mitogen-activated protein kinase genes in defense response (Liu et al., 2011), it is poorly seed transmitted in soybean (Krell et al., 2003), whereas TSV that I used in this study has seed transmission rates as high as 95% in some cultivars (e.g., PI88799). CMV (Nagamatsu et al., 2007) and *Apple latent spherical virus* (ALSV) (Yamagishi and Yoshikawa, 2009)-based VIGS have been used in soybean to silence genes in the flavonoid pathway in seeds and seedlings. The CMV and the ALSV vectors use a T7-promoter that requires *in vitro* RNA transcription before inoculation of plants. However, the TSV-based vector that I developed here is DNA based with a *Cauliflower mosaic virus* (CaMV) 35S promoter that can be directly introduced into plants which improves the efficiency and hence making it more suitable to high throughput applications (Zhang et al., 2009).

Large inserts of 1.3 kb have been successfully used in *Tobacco rattle virus* (TRV)-based VIGS in *Nicotiana bentamiana* and short inserts of 76 base pairs were sufficient for efficient silencing

in *Arabidopsis thaliana* using a *Turnip yellow mosaic virus* (TYMV) VIGS vector (Liu and Page, 2008; Pflieger et al., 2008). The upper limit for the size of the insert is dependent on the virus and the host plant. Larger inserts can inhibit systemic spread of the virus and can also result in the loss of the inserted gene from the virus vector (Burch-Smith et al., 2004). Complementarity of at least 23 nt to the target gene was essential for effective gene silencing in *N. benthamiana* using a *Potato virus X* (PVX) VIGS vector (Thomas et al., 2001). Inverted-repeat gene inserts of 40 - 60 bases increased the efficiency of silencing in *Tobacco mosaic virus*-, *Barley stripe mosaic virus*- and TYMV-based vectors, possibly by the silencing triggered by the as hairpin structures in the RNA transcribed from inverted repeats (Lacomme et al., 2003; Pflieger et al., 2008). Here, I examined the effect of different sizes of gene inserts and inverted repeats on silencing.

VIGS-based degradation of target transcript is caused by plant virus induced RNA silencing (Llave, 2010). Gene silencing was first described in plants as co-suppression, where introduction of multiple copies of a gene resulted in lower gene expression because of reduced mRNA accumulation (Napoli et al., 1990). The role of double-stranded (ds)RNA in induction of PTGS was later identified (Fire et al., 1998). The genomes of about 90% of plant pathogenic viruses are composed of ssRNA and replicate by producing dsRNA intermediates (Waterhouse et al., 2001). The plant detects and degrades dsRNA to counter virus infection and systemic spread in the plant (Angell and Baulcombe, 1997; Waterhouse et al., 2001). Plant defense systems processes the viral dsRNA into 21-24 nucleotide (nt) small interfering (si)RNAs (Hamilton and Baulcombe, 1999; Hamilton et al., 2002).

Replicating viral dsRNAs or the secondary hairpin structures in the virus genome are the likely targets that are processed by Dicer-like 2 (DCL2), DCL3 and DCL4 into 22 nt, 24 nt and 21 nt primary virus-derived (v)siRNA duplexes, respectively (Fusaro et al., 2006). DCLs are RNase III-like enzymes that bind to and cleave dsRNA producing smaller dsRNA products with 5' terminal monophosphate group and a 2 nt 3' terminal overhang. DCL4 plays a major role in silencing viruses and DCL2 plays a subsidiary role, whereas DCL3, which produces 24 nt vsiRNA does not play a major role in antiviral defense (Blevins et al., 2006; Deleris et al., 2006; Garcia-Ruiz et al., 2010). DCL1, involved in microRNA processing (Kurihara et al., 2006), can suppress virus silencing by negatively regulating the expression of DCL3 and DCL4 (Qu et al., 2008).

The siRNA duplexes are the mobile signals that spread silencing through the plant and defend the plant from virus infection (Dunoyer et al., 2010). The vsiRNA duplex undergoes methylation at the 2' OH of the 3' terminal nucleotide by the HUA ENHANCER1 (HEN1) protein that protects it from degradation (Li et al., 2005; Yang et al., 2006). Methylated siRNA duplexes are loaded into Argonaute (AGO) protein, a RNase H like enzyme, and a single strand of the siRNA duplex is retained and the complementary strand is degraded forming RNA-induced silencing complexes (RISCs) (Qi et al., 2005). RISCs, comprised of siRNA bound to AGO, target complementary ssRNA leading to RNA cleavage or translation repression (Baumberger and Baulcombe, 2005; Brodersen et al., 2008). The AGO1, AGO2 and AGO7 are involved in silencing of viruses in plants (Harvey et al., 2011; Jaubert et al., 2011; Morel et al., 2002; Qu et al., 2008).

Plant RdRps 1 (RDR1), RDR2 and RDR6, amplify silencing signals by producing secondary vsiRNA (Garcia-Ruiz et al., 2010; Qu et al., 2008; Wang et al., 2010). DCL4-dependent 21 nt vsiRNAs were necessary for production of secondary vsiRNAs in plants (Wang et al., 2011). RDR6 has also been implicated in the prevention of the entry of viruses into the meristem by PVX (Schwach et al., 2005).

Plant viruses counteract plant antiviral defenses by expressing proteins that suppress RNA silencing by targeting the silencing pathway at distinct steps (Voinnet et al., 1999). The P38 protein of *Turnip crinkle virus* suppresses RNA silencing by binding to long dsRNA or siRNA duplexes and P1/HC-Pro of TEV sequesters only siRNA duplexes to suppress silencing (Anandalakshmi et al., 1998; Merai et al., 2006). P1/HC-Pro can also inhibit the 3' terminal methylation of vsiRNA duplexes reducing the formation of RISCs targeted against the virus genome (Ebhardt et al., 2005). HC-Pro from *Turnip mosaic virus* has been shown to inhibit RDR-based secondary siRNA production (Moissiad et al., 2011). The 2b silencing suppressor of CMV interacts with AGO1 and suppresses cleavage of RNA by AGO1. The 2b protein also blocks long-distance movement of mobile silencing signals (Guo and Ding, 2002; Zhang et al., 2006). In TSV, there has been no report of a silencing suppressor, although the 2b protein has been suggested by relation to the 2b protein of CMV to be involved in suppressing silencing (Brigneti et al., 1998; Xin et al., 1998). The nearly complete symptom recovery in TSV-infected soybean plants indicates that TSV expresses a weak suppressor of PTGS making TSV an ideal starting point for construction of a VIGS vector to study soybean meristem and seed development.

Virus silencing suppressors also interfere with micro RNA production and activity (Kasschau et al., 2003). Micro (mi)RNAs are plant endogenous small RNAs of 21-24 nt that are processed from stem-loop regions of transcripts by DCL1 in the nucleus (Kurihara and Watanabe, 2004; Papp et al., 2003). The miRNA/miRNA* strand is methylated by HEN1 and the miRNA is loaded into AGO1 to form the RISC complex that targets complementary transcripts for cleavage or translational repression (Brodersen et al., 2008; Vaucheret et al., 2004; Yu et al., 2005). HASTY (HST), homologous to Exportin-5, is involved in the transport of the miRNA from the nucleus to the cytoplasm (Bollman et al., 2003; Lund et al., 2004). Virus silencing suppressors, P1/HC-Pro (TuMV, *Turnip mosaic virus*), p19 (*Tomato bushy stunt virus*) and p12 (*Beet yellow virus*), can inhibit mRNA cleavage by miRNA by inhibiting the degradation of miRNA* strand (Chapman et al., 2004). Induction of miR168, that down-regulates AGO1, was recorded during virus infection and by virus silencing suppressors in plants (Varallyay et al., 2010).

The influence of virus infection on siRNA populations in plants have been investigated in cotton infected by *Cotton leafroll dwarf virus* and rice infected by *Rice stripe virus* using small RNA deep sequencing (Silva et al., 2011; Yan et al., 2010). Deep sequencing of siRNA has also been used to understand the role of RDR and DCL in virus infection in *Arabidopsis* (Garcia-Ruiz et al., 2010; Qi et al., 2009). Here I examined the siRNA and transcriptome profiles using small RNA deep sequencing and mRNA-Seq, respectively, in mock-inoculated and TSV-infected soybean plants using Illumina sequencing.

OBJECTIVES

The overall objectives were to identify the role of SMV genes in seed and aphid transmission in soybeans and to design and implement a DNA-based VIGS system using TSV for functional genomics of soybean seed development. The specific objectives were to:

- Study the role of HC-Pro, CP and amino acid residues within CP in seed and aphid transmission of SMV in soybean.
- Evaluate the role of P1 and NIb in seed and aphid transmission in SMV in soybean.
- Design a TSV DNA-based vector for VIGS in soybean.
- Determine the length of the gene fragments and inverted repeat sequence inserts for effective gene silencing using the TSV-based VIGS vector.
- Detect changes in siRNA and miRNA populations in TSV-infected soybean.
- Identify transcriptome changes in TSV-infected plants.

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FIGURES

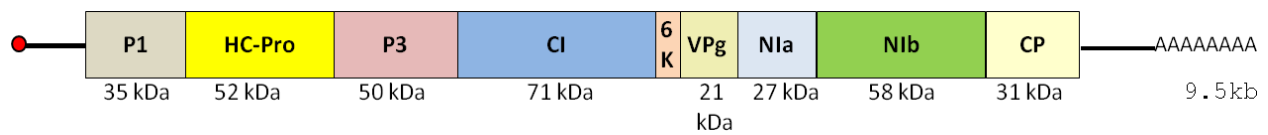


Figure 1.1. Genome organization of *Soybean mosaic virus* (SMV), a potyvirus. The genomic RNA (9.5kb) encodes a large polyprotein that is proteolytically processed into P1, helper component proteinase (HC-Pro), P3, cylindrical inclusion (CI) protein, 6K (6 kDa), viral genome-linked protein (VPg), nuclear inclusion proteins (NIa and NIB), and coat protein (CP) (Adams et al., 2005; Ng and Falk, 2006).

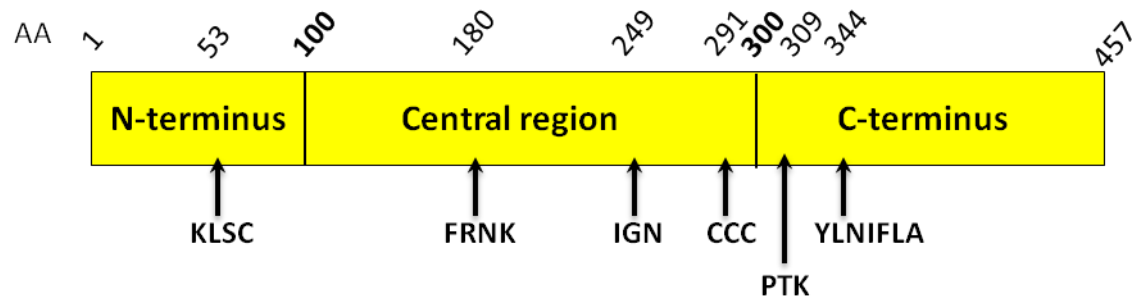


Figure 1.2. The helper component proteinase (HC-Pro) of *Soybean mosaic virus* has been divided into three functional domains based on similarities to HC-Pro of other potyviruses with the conserved amino acid motifs (below) and the corresponding amino acid positions (above) indicated.

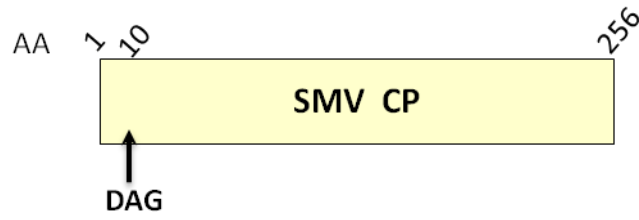


Figure 1.3. *Soybean mosaic virus* (SMV) coat protein (CP) consists of 256 amino acid residues with the DAG amino acid motif at position 10.

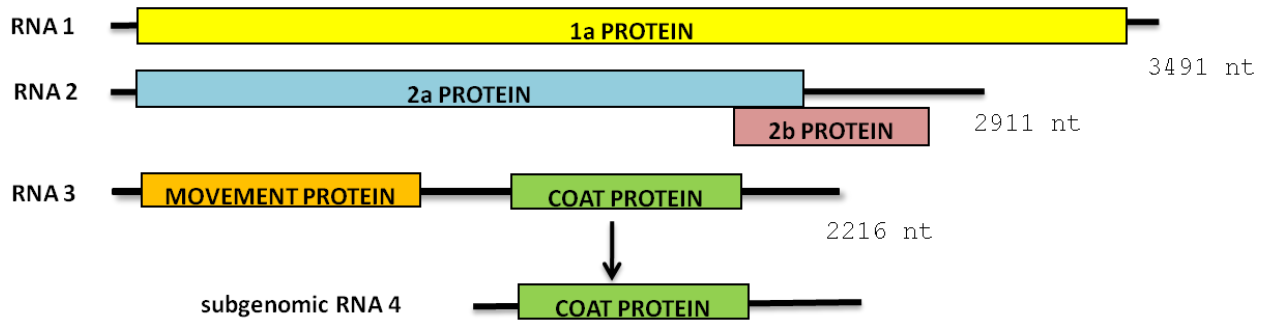


Figure 1.4. Genome organization of *Tobacco streak virus* (TSV) with RNA1 of 3491 nt encoding 1a protein (Replicase). RNA2 is 2911 nt in length and encodes two proteins, 2a (RNA-dependent RNA polymerase) and 2b, from overlapping open reading frames. RNA3 is 2216 nt in length and contains open reading frames for the movement and coat proteins, but expresses only the movement protein. The subgenomic RNA4 is 3' co-terminal with RNA3 and encodes the coat protein, which is required for TSV infection.

CHAPTER 2

ROLE OF *SOYBEAN MOSAIC VIRUS* GENES IN SEED AND APHID TRANSMISSION IN SOYBEAN

ABSTRACT

Soybean mosaic virus (SMV) is a seed and aphid transmitted member of the family *Potyviridae* that can cause significant yield reductions and lower seed quality in soybean, *Glycine max* (L.) Merr. In North America, seed transmission serves as the primary source of inoculum for SMV as there are very few alternative hosts for the pathogen and the secondary spread of the virus is by aphids. The roles in seed and aphid transmission of SMV encoded proteins, P1, helper component protease (HC-Pro), nuclear inclusion b (NIb) and coat protein (CP), were investigated by constructing chimeric recombinants between SMV 413 (efficiently aphid and seed transmitted) and SMV G2 (not aphid or seed transmitted). Seed transmission of SMV was influenced by P1, HC-Pro, and CP. Mutation of the amino acid G at position 12 to D in the DAG amino acid motif of the CP, and the Q amino acid residue at position 264 to P near the C-terminus of the CP, affected seed transmission. The DAG motif in the CP was also the major determinant in aphid transmission and HC-Pro played a secondary role as evidenced by the aphid transmission of the non-aphid transmitted SMV G2, by replacing the HC-Pro with the SMV 413 HC-Pro and with D12 to G mutation in the CP. The severity of the foliar symptoms was altered by exchanging the HC-Pro coding regions between the two SMV isolates. Seed coat mottling was linked to a single amino acid change of Q to P at position 264 in the CP of SMV 413.

INTRODUCTION

Soybean mosaic virus (SMV) is a seed and aphid transmitted virus that infects soybean, *Glycine max* (L.) Merr., that can cause yield reductions as high as 35% and has been reported worldwide from all soybean growing areas (Hill, 1999). SMV infection in soybean can lead to extensive seed coat mottling and reduced seed size and weight, with low seed oil content resulting in poor seed quality (Hill, 1999; Hobbs et al., 2003). SMV is a member the genus *Potyvirus* (family *Potyviridae*), one of the largest genera of plant viruses (Gibbs and Ohshima, 2010). SMV infected seeds serve as the primary source of inoculum in the field as there are very few alternative hosts of the virus in North America (Hill, 1999).

Seed transmission of 0 to 43% has been recorded with specific SMV strain to soybean line interaction required for seed transmission (Domier et al., 2007). Seed transmission of SMV in soybean has been linked to the chromosomal regions that contain homologues of Dicer-like 3 and the RNA-dependent RNA polymerase 6 genes that are involved in antiviral defense through RNA silencing (Domier et al., 2011). The P1/ helper component proteinase (HC-Pro) of *Tobacco etch virus* (genus *Potyvirus*) is implicated in suppression of post-transcriptional gene silencing (Kasschau and Carrington, 2001). P1/HC-Pro interferes with gene silencing by binding to small interfering (si)RNA duplexes, inhibiting the 3'-terminal methylation of siRNA duplexes and by suppressing secondary siRNA production (Ebhardt et al., 2005; Merai et al., 2006; Moissiad et al., 2011). P1 can also suppress silencing by directly binding to argonaute (AGO) protein in RNA-induced silencing complexes (RISCs) (Giner et al., 2010). In *Pea seed-borne mosaic virus* (PSbMV) (genus *Potyvirus*) HC-Pro, which is expressed from the 5' half of the virus genome,

and nuclear inclusion body a (NIa) and b (NIb) proteins, which are expressed from the 3' half of the virus genome, were associated with seed transmission. However, the P1 region showed no influence on seed transmission (Johansen et al., 1996). HC-Pro is a multifunctional protein associated with virus movement, aphid transmission and gene silencing suppression (Blanc et al., 1997; Blanc et al., 1998; Cronin et al., 1995; Rojas et al., 1997; Varrelmann et al., 2007). NIa protein is a serine protease, which is one of the three endopeptidases encoded by potyviruses that processes their polyproteins (Carrington et al., 1993; Hellmann et al., 1988). NIb is the RNA-dependent RNA polymerase linked to virus replication (Domier et al., 1987).

In PSbMV, infection of the embryo and suspensor cells occurs before maturation from the endosperm through transient vesicles at the base of the suspensor cells at the micropylar region (Robert et al., 2003). PSbMV invades the endosperm through plasmodesmata from the testa cells. The presence of the virus in the micropylar region before the degeneration of the transient vesicles in the suspensor was necessary for embryo invasion (Robert et al., 2003). In SMV, survival of the virus within the maturing embryo also plays an important role in seed transmission (Bowers and Goodman, 1979).

Spread of SMV in the field is primarily by non-colonizing aphids in a nonpersistent manner (Hill, 1999). There are conflicting evidences on the role of *Aphis glycines* Matsumura, a colonizing aphid on soybean, on the spread of SMV in the field in the US. Reports from Wisconsin showed a clear association of the *A. glycines* populations with SMV spread in 2001 and 2002 (Burrows et al., 2005), whereas reports from Iowa and Wisconsin in 2004 and 2005 indicated that the *A. glycines* did not contribute to the incidence of SMV infections in the field

(Pedersen et al., 2007). Aphid transmission of most potyviruses has been proposed to be depended on the interaction between HC-Pro and the coat protein (CP) (Blanc et al., 1997; Dombrovsky et al., 2005; Domier et al., 2003; Flasiński and Cassidy, 1998; Seo et al., 2010). The KLSC amino acid sequence motif near the N-terminus of SMV HC-Pro has been implicated in binding to *A. glycines* stylets (Seo et al., 2010) and second amino acid sequence motif, PTK, in the HC-Pro of *Zucchini yellow mosaic virus* has been implicated in binding to virus CP (Peng et al., 1998). However, the PTK motif of SMV failed to influence HC-Pro interactions with CP, although it was important in aphid transmission (Seo et al., 2010). The DAG amino acid sequence motif of the CP, a conserved motif in most potyviruses, binds to the HC-Pro transiently during aphid transmission (Atreya et al., 1990; Blanc et al., 1997; Lopez-Moya et al., 1999). The DAG motif of SMV has also been linked to aphid transmission by *A. glycines* (Seo et al., 2010). Several other conserved amino acid residues in HC-Pro and CP of specific potyviruses have been identified to be linked to aphid transmission (Dombrovsky et al., 2005; Flasiński and Cassidy, 1998; Llave et al., 2002; Seo et al., 2010).

Seed coat pigmentation of soybean is governed by alleles at the *I* locus that regulate chalcone synthase (CHS) mRNA levels by post-transcriptional gene silencing (Senda et al., 2004; Todd and Vodkin, 1996; Tuteja et al., 2009). Chalcone synthase is an important enzyme in the flavonoid pathway that synthesizes anthocyanin and proanthocyanidin pigments. In some soybean genotypes, the *I* locus contains multiple copies of CHS genes organized into perfect inverted repeats (Clough et al., 2004). The siRNAs produced from these inverted repeats silences CHS gene expression that results in yellow, instead of black, pigmented seeds (Clough et al., 2004; Tuteja et al., 2009). In soybean infected with SMV, seeds with SMV-induced seed coat

mottling had higher CHS mRNA levels in the seed coat than the uninfected seeds, which were attributed to the suppression of gene silencing by SMV (Senda et al., 2004).

Here, I examined the role of SMV proteins in seed and aphid transmission of the virus in soybean. The effect of proteins P1, HC-Pro, NIb and CP; and amino acid residues within CP on seed and aphid transmission were tested. The effects of the virus proteins and amino acid residues on foliar symptoms and seed coat mottling were also investigated.

MATERIALS AND METHODS

Virus isolates and plant materials

The field isolate SMV 413 that can be seed transmitted at a rate of up to 32% in plant introduction (PI) 68671, a laboratory strain SMV G2 that shows no seed transmission in ‘PI 68671’ (Domier et al., 2007) and recombinants between the two unmodified viruses were used to study seed transmission. The role of SMV genes in aphid transmission were also investigated with the recombinants constructed between SMV 413 and SMV G2, which showed aphid transmission rates of 67% and 0%, respectively, in soybean cultivar ‘Williams 82’.

A full-length infectious clone of SMV 413 in pCR TOPO4 (Invitrogen, Carlsbad, CA) was developed in our lab (Domier et al., 2011) and strain G2 in pBR322 was provided by J. Hill (Iowa State University, Ames). pCR TOPO4 is a high copy number plasmid with a kanamycin resistance selectable marker and SMV 413 was cloned downstream of a T7 RNA polymerase

promoter. The plasmid vector, pBR322, is a medium copy-number plasmid with selectable markers of ampicillin/carbenicillin and tetracycline. SMV G2 was cloned downstream of a *Cauliflower mosaic virus* (CaMV) 35S promoter in pBR322.

The virus inoculum for SMV 413 was prepared from systemically infected leaves of ‘Williams 82’ that had been mechanically inoculated at the unifoliate stage with capped *in vitro* transcripts. The *in vitro* transcripts were synthesized with the mMESSAGE mMACHINE Kit (Ambion, Austin, TX) from *SmaI* digested purified linear SMV 413 cDNA clones (13.3 kb) (Domier et al., 2011). The capped transcripts (20 µl) were mixed with an equal volume of 6 mM sodium phosphate buffer (pH 9.0) containing 1 mg of bentonite and mechanically inoculated on Carborundum- (320 grit, Fisher, Fairlawn, NJ) dusted unifoliate leaves. The inoculated plants were rinsed in sterile water after 5 min and maintained in the greenhouse for symptom development. The SMV G2 clone (14.5 kb) was biolistically inoculated using the Helios Gene Gun system (Bio-Rad, Hercules, CA, USA) as recommended by the manufacturer with 1.6 µm gold particle at a rate of at least 0.125 mg gold particle/shot and 1 µg of plasmid DNA/shot (Yamagishi et al., 2006). ‘Williams 82’ seedlings at the unifoliate stage were bombarded with 5 shots per plant using helium at 1.24 MPa on the adaxial leaf surface. The plants were then sprayed with water and covered with plastic bags and placed overnight (12 to 18 h) in dim light and then the bags were removed and the plants were maintained in the greenhouse at temperatures ranging from 25°C to 27°C and a photoperiod of 12 h.

Seeds of ‘PI 68671’ were obtained from the USDA Soybean Germplasm Collection, Urbana, IL. The aphid transmission studies were conducted using virus free soybean, ‘Williams 82’, obtained

from Missouri Foundation Seed, University of Missouri, Columbia, MO. Soybean plants were grown in 2:1 soil:soilless (Metro-Mix 900, Sun Gro Horticulture Inc., Bellevue, WA) mix.

Construction of chimeric SMV clones

Mutant clones were developed to investigate the role of SMV proteins in seed and aphid transmission. Unique restriction sites present in both the full-length cDNA clones of SMV 413 and SMV G2, or present in one of the clones, were identified and manipulated to construct the chimeric clones. The mutant viruses, pG4-Sal and p4G-Sal, were constructed using the unique *SalI* restriction enzyme sites present at nucleotide (nt) position 3,786 in SMV 413 and nt 3,782 in SMV G2 (Fig. 2.1) and *NotI* sites present in the plasmid vectors at nt positions 13,109 and 13,597 in SMV 413 and SMV G2 clones, respectively. The SMV clones were sequentially digested with *SalI* and *NotI* and the *NotI-SalI* fragment from the 5' terminus of the SMV G2 was ligated (T4 DNA ligase, Promega Corp., Madison, WI) with the *NotI-SalI* fragment from the 3' terminal region of the SMV 413 and vice versa to make pG4-Sal and p4G-Sal, respectively (Fig. 2.2). Ligation reactions were electroporated into electrocompetent *Escherichia coli* (DH10B), screened on Luria-Bertani (LB) plates containing ampicillin or kanamycin and amplified (Sambrook and Russel, 2001). pG4-Sal was biolistically bombarded into unifoliate leaves of soybean. p4G-Sal was inoculated into soybean using capped *in vitro* transcripts synthesized from cDNA clones linearized with *ClaI*. The construction of clones, pG4-Sal and p4G-Sal, were confirmed by restriction digestion with *HindIII* and by sequencing with primer 3565F-SMV (Table 2.1) with the Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA) at the University of Illinois, W. M. Keck Center for Comparative and Functional Genomics.

The role of HC-Pro in seed and aphid transmission was examined by reciprocally exchanging the coding sequence for HC-Pro between SMV G2 and SMV 413 to produce mutant SMV clones pG2-HPmut and p413-HPmut, respectively (Fig 2.2). These clones were developed using a unique *SacII* restriction site present near the 5' terminus of the HC-Pro coding region in SMV 413 and *BlpI* sites present near the 3' termini of the HC-Pro coding region in both full-length cDNA clones (Fig. 2.1)

The pG2-HPmut was developed by reverse PCR of SMV G2 using primers G2Hc-BlpI3050F (5'-TAAAACGCGTGCTGAGCTCCCACGCATTTTGGTTG-3'; *MluI* and *BlpI* sites underlined) and G2Hc-SacII1857R (5'-TAAAACGCGTCCGCGGAAAACTGAGCTTCAGGAG-3'; *MluI* and *SacII* sites underlined) to synthesize a product of 13.1 kb. The inverse PCR using iProof High Fidelity (HF) DNA Polymerase (Bio-Rad, Hercules, CA), a proof-reading DNA polymerase, was performed at reaction conditions of 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 69°C for 30 s, and 72°C for 8 min, with a final extension period of 72°C for 10 min. The PCR product was digested with *MluI* and ligated to form pG2-ΔHP, a SMV G2 clone with the HC-Pro coding sequence deleted. After confirmation of pG2-ΔHP by *MluI* digestion, the plasmid was digested with *SacII* and *BlpI*. The *SacII-BlpI* SMV 413 fragment of 1.2 kb comprising the HC-Pro coding region was ligated to the *SacII-BlpI*-modified SMV G2 fragment (13.1 kb), transformed into *E. coli* and the construction was confirmed by *HindIII* digestion and sequencing, using primer 964F-SMV (Table 2.1). The pG2-HCmut clone was bombarded into soybean plants as described for SMV G2.

p413-HPmut was constructed by inserting a *Sac*II site at the 5' terminus of the coding sequence of HC-Pro of SMV G2 by PCR, using forward primer G2-HcPro-SacIIF (5'-GAGACCCGCGGTTGGAAAAAGGTGTTTGAT-3'; *Sac*II site underlined) and the reverse primer G2-HcPro-BlpIR (5'-GAGAGCTCAGCATTCCTGGTTTCAGGGTGA-3'; *Blp*I site underlined) with iProof DNA Polymerase. The PCR was conducted at 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 62°C for 30 s, and 72°C for 1 min, with a final extension period of 72°C for 10 min. SMV 413 and the PCR-amplified SMV G2 HC-Pro coding region were digested with *Sac*II and *Blp*I. The fragment of SMV 413 lacking the HC-Pro coding region (12.1 kb) was gel purified with a QIAquick Gel Extraction Kit (Qiagen Inc. Valencia, CA) and ligated to the *Sac*II-*Blp*I fragment (1.2 kb) of SMV G2 HC-Pro. The ligation reaction was transformed into *E. coli* and verified by sequencing with 964F-SMV (Tables 2.1). Capped *in vitro* transcripts of the clone were inoculated into soybean seedlings as described for SMV 413.

The influence of Nib protein on seed and aphid transmission was studied with p413-NIbmut, which was constructed by introducing *Bgl*III and *Ppu*MI sites near the 5' and the 3' termini of the Nib coding region of SMV G2 (Fig. 2.1, 2.2). The *Bgl*III-*Ppu*MI fragment also includes the DAG/DAD motif from the N-terminus of the CP. The *Bgl*III and *Ppu*MI restriction enzyme sites were inserted in SMV G2 with forward primer SMVG26954BglIIF (5'-GAGAAGATCTCTTTGGAAACACAGTGA-3'; *Bgl*III site underlined) and the reverse primer SMVG28556PpuMIR (5'- GAGAGGTCCTTATCTGCATCCATATCT-3', *Ppu*MI site underlined). PCR with iProof HF DNA polymerase was carried out at 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 64°C for 30 s, and 72°C for 1 min 30 s, with a final extension period of 72°C for 10 min. SMV 413 and the PCR product of G2 Nib, which included the DAD motif

were digested with *Bgl*II and *Ppu*MI. The SMV 413 fragment (11.7 kb) lacking the NIb coding sequence was gel purified and ligated to the *Bgl*II-*Ppu*MI cleaved NIb coding region of SMV G2. The ligation reaction was transformed, screened and capped transcripts were inoculated on to soybean plants as described for SMV 413. The recombinant clones were confirmed by *Bam*HI digestion and sequencing with 6551F-SMV (Tables 2.1).

To determine the significance of P1 protein in seed and aphid transmission, p413-P1mut was constructed by exploiting the *Sac*II site present in SMV 413 (Fig 2.1, 2.2) similar to p413-HPmut and p413-NIbmut. Clones pG2- Δ HP and SMV 413 were digested with *Not*I (site present in the cloning vectors) and *Sac*II (site present in both the virus clones). The 10 kb fragment of *Not*I-*Sac*II SMV 413 lacking P1 coding sequence and the 1.8 kb *Not*I-*Sac*II pG2- Δ HP containing the P1 coding region of SMV G2 were gel purified, ligated, transformed into *E. coli*, and verified by *Hpa*I digestion and sequencing with 964F-SMV (Table 2.1). p413-P1mut was bombarded into soybean plants as described for SMV G2.

The conserved DAG amino acid motif present proximal to the N-terminus of CP in most potyviruses has been implicated in aphid transmission and interaction with the HC-Pro (Blanc et al., 1997; Seo et al., 2010). SMV G2, which is not aphid or seed transmitted, contains a DAD rather than DAG motif (Fig. 2.3). The role of this motif and its interaction with HC-Pro in aphid and seed transmission was investigated using two sets of mutant clones. First, the DAG motif was changed to a DAD motif in SMV 413 and p413-HPmut to produce p413-CPmut and p413-DM, respectively, by site-directed mutagenesis. The p413-CP1 clone was developed by substituting nucleotides G and C at positions 35 and 36 of the CP coding region of SMV 413

with AT (Fig. 2.3A), changing the amino acid position 12 from G to D (Fig. 2.3B; 2.5). Overlap-extension PCR, which comprises of 3 PCR amplification, with two overlapping primers containing the base changes of GC to AT (Higuchi, 1990) was used with iProof DNA polymerase for mutagenesis. PCR-1 used SMV 413 as a template with primer SMV413CP1-*Bgl*IIIF (5'-GAGAGATCTCTTTGGAAATACAGTGAC-3'; *Bgl*III site underlined) and SMV413CP2-mut1R (5'-TGGGTCCTTATTCTGCATCCATGT-3'; AT mutation underlined). PCR-2 used SMV 413 as a template with primers SMV413CP3-mut2F (5'-ACATGGATGCAGATAAGGACCCA-3'; AT mutation underlined) and SMV413CP4-*Mlu*IR (5'-GAGACGCGTTAGAACTCAAGCTAT-3'; *Mlu*I site underlined). Conditions for PCR-1 and PCR-2 were 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 63°C for 30 s, and 72°C for 30 s, with a final step of 72°C for 10 min. PCR-3 with 1 ng each of PCR-1 and PCR-2 products as template and primers SMV413CP1-*Bgl*IIIF and SMV413CP4-*Mlu*IR generated a 2.8 kb product with *Bgl*III and *Mlu*I sites at the 5' and 3' termini, respectively, and the GC-to-AT mutation at positions 35 and 36 in the CP of SMV 413. The extension time for PCR-3 was extended to 1 min at 72°C. The PCR-3 product and SMV 413 were digested with *Bgl*III and *Mlu*I present at 6957 and 9713 nt in SMV 413, respectively (Fig. 2.1). The 10-kb fragment after *Bgl*III-*Mlu*I digestion of SMV 413 was ligated to the *Bgl*III-*Mlu*I PCR-3 product to synthesis the p413-CP1mut (Fig. 2.5). The ligation reaction was then transformed and colonies were screened for G12 to D mutation in the DAG motif by sequencing with 8238F-SMV (Tables 2.1). To construct p413-DM the *Bgl*III-*Mlu*I PCR-3 product was ligated into *Bgl*III-*Mlu*I cleaved p413-HPmut (Fig 2.5). The nucleotide changes in the clone were confirmed by sequencing with 964F-SMV and 8238F-SMV (Table 2.1). Plants were inoculated with p413-CP1mut and p413-DM as described for SMV 413.

A second set of mutations was constructed where the DAD amino acid sequence motif was mutated to DAG in SMV G2, p4G-Sal and pG2-HPmut by overlap-extension PCR to produce pG2-CPmut, p4G-Sal-CP, and pG2-DM, respectively. PCR-1 used SMV G2 cDNA clone as the template and primers SMVG2CP1-AgeIF (5'-GAGAACCGGTGTGTGCGTTACAACAA-3'; *AgeI* site underlined) and SMVG2CP2-mut1R (5'-CTTTGGATCCTTGCCTGCATCCATATC-3'; GC mutation underlined). PCR-2 used SMV G2 as a template with primers SMVG2CP3-mut2F (5'-GATATGGATGCAGGCAAGGATCCAAAG-3'; GC mutation underlined) and SMVG2CP4-NheIR (5'-GAGAGCTAGCTCTCTATCTCTCAAATTCC-3'; *NheI* site underlined). PCR-3 was performed using primers SMVG2CP1-AgeIF and SMVG2CP4-NheIR and 1 ng each of PCR-1 and PCR-2 as template. The PCR conditions for PCR-1 and PCR-3 were 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 2 min, with a final step of 72°C for 10 min, the PCR-2 had a similar thermal cycle but with a DNA extension duration of 15 s at 72°C. The PCR-3 product (4.5 kb) and SMV G2 were digested with *AgeI* (Fig. 2.1.) and *NheI* (9115 nt), and the 9.8 kb SMV G2 fragment was ligated to the *AgeI-NheI* PCR-3 product to develop pG2-CPmut containing the DAG motif in CP (Fig 2.5). The ligation reaction was transformed, screened and the mutation was confirmed by sequencing with 8238F-SMV. In p4G-Sal-CP (Fig 2.5) was constructed by site-specific mutagenesis of p4G-Sal using the same primers and restriction sites for overlap-extension PCR as used for pG2-CPmut. The p4G-Sal-CP had the SMV 413 at the 5' terminus and SMV G2 at the 3' terminus and was developed exploiting the common *SalI* site as in p4G-Sal but with the DAG motif in the CP. Construction of p4G-Sal-CP was confirmed by sequencing with 3565F-SMV and 8238F-SMV (Table 2.1). Plants were inoculated with p4G-Sal-CP as described for SMV 413. pG2-DM (Fig

2.5) was synthesized with pG2-HPmut as template and the same primers as pG2-CPmut by overlap-extension PCR using the *Age*I and *Nhe*I restriction sites. The mutation at the DAG motif was confirmed by sequencing with 964F-SMV and 8238F-SMV (Table 2.1). Plants were inoculated with SMV G2-CP, p4G-SaI-CP and pG2-DM as described for SMV G2.

The single amino acid mismatch of Q to P at position 264 of the two SMVs is the only amino acid residue other than the DAG motif that differs in the CPs of SMV 413 and SMV G2. To investigate the role of this amino acid in seed and aphid transmission the amino acid Q was mutated to P with a single nucleotide change from A in SMV 413 to C in SMV G2 (Fig 2.4) to develop the p413-CP2mut (Fig 2.5) . The substitution of A to C at nucleotide position 791 of the CP in SMV 413 was carried out by overlap-extension PCR with three PCR reactions using the *Bgl*II and *Mlu*I sites as in p413-CP1mut to insert the mutated fragment. The PCR-1 for p413-CP2mut was carried out with the same forward primer as in p413-CP1mut (SMV413CP1-BglIIIF) with the *Bgl*II site and reverse primer SMV413CP2-mut1R (5'-TCTTTACTGCGGTGGGCCCAT-3'; G mutation underlined). Conditions for PCR-1 were 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 63°C for 30 s, and 72°C for 1 min, with a final step of 72°C for 10 min. PCR-2 was performed with forward primer SMV413CP2-mut2F (5'-ATGGGCCACCGAGTAAAGA-3'; C mutation underlined) and the reverse primer SMV413CP4-MluIR with the *Mlu*I site used in the synthesis of p413-CP1mut clone. The PCR-2 thermal cycle was similar to PCR-1 except for shorter duration of extension of 30 s at 72°C. PCR-1 and PCR 2 were done with the SMV 413 template and the PCR-3 had a template consisting of the mix of 1 ng each of PCR-1 and PCR-2 products. The PCR-3 with SMV413CP1-BglIIIF and SMV413CP4-MluIR was conducted at the same conditions as p413-

CP1mut. The clones were digested, ligated, transformed, screened and inoculated following the same method as in p413-CP1mut and was verified by sequencing with 9455R-SMV (Table 2.1). To further investigate the interactions between both the amino acid mismatches in the CP of SMV 413 and SMV G2 CP (Fig 2.3; 2.4) on seed and aphid transmission, p413-CP-DM was constructed with both the amino acid mutations of G12 to D and Q264 to P (Fig 2.5). p413-CP-DM was constructed using overlapping-extension PCR with primers SMV413CP1-BglIII, SMV413CP2-mut1R, SMV413CP2-mut2F and SMV413CP4-MluI on p413-CP1mut template. p413-CP-DM was processed by the same protocol as p413-CP2mut and the construction was confirmed by sequencing with 8238F-SMV and 9455R-SMV (Table 2.1).

Seed transmission

The influence of SMV genes on seed transmission rates was investigated with SMV 413, SMV G2 and the thirteen SMV mutants in soybean cultivar 'PI 68671'. The 'PI 68671' seeds were germinated and seedlings were tested for SMV infection using tissue blot immunoassay (TBIA) prior to seed transmission studies (Lin et al., 1990). The tissue samples for TBIA were drawn from unifoliate leaf using a wooden applicator dipped in phosphate buffered saline (PBS, 0.02M K₂HPO₄ and 0.15 M NaCl, pH 7.4) on the polyethylene side of a clean Benchkote protector sheet (Whatman Scientific Ltd, Maidstone, UK). The tip of the wooden applicator coated with tissue sample was smeared on 82 mm nylon membrane disc (Amersham Hybond-N, Little Chalfont, UK). The membrane was washed in PBS with 5% Triton X-100 for 10 min at room temperature and the membrane was wiped with Kimwipes (Kimberly-Clark Corp, Irving, TX) soaked in Triton X-100 to remove any excess tissue. The membrane was transferred into 10

ml blocking solution (BS, 1% nonfat dry milk in PBS) and incubated for at least 30 min at room temperature. Alkaline phosphatase enzyme conjugate for SMV (Agdia, Inc., Elkhart, IN) was added to the BS at 1:1000 dilutions and incubated for 2 h at room temperature. The membrane was washed three times in PBS-Tween (0.05% Tween 20 in PBS) for 10 min each. The membrane was then washed in substrate buffer (0.5 mM magnesium chloride hexahydrate and 1M diethanolamine in water at pH 9.8) and transferred to Western Blue stabilized substrate for alkaline phosphatase (Promega Corp, Madison, WI) and incubated for 30 minutes for color development of the positive sample. The TBIA was conducted in 2 replications and tissue samples from healthy plants as negative control and tissue from identified SMV infection as positive control were included in the testing.

Confirmed healthy 'PI 68671' seedlings were mechanically inoculated for seed transmission at V2 stage (Fehr et al., 1971) using systemically infected 'Williams 82' with each unmodified isolate/mutant SMV clone. The plant extracts for the mechanical inoculations were prepared in 10 mM sodium phosphate buffer (pH 7.0) and were inoculated by rubbing with pestles dipped in the extract on to Carborundum (Fisher) dusted foliage. Infection of plants with the intended unmodified/mutant SMV isolate was confirmed by sequencing reverse transcription (RT)-PCR products from inoculated plants. Total RNA was extracted from 100 mg of plant tissue using RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA). First-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) using appropriate primers (Table 2.1). The first-strand cDNA was amplified for sequencing using primers specific to the site of recombination of the SMV chimeras (Table 2.1). PCR with GoTaq Green Master Mix (Promega Corp, Madison, WI) was done by initial denaturing of DNA at 95°C for 2 min

followed by 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 2 min, with a final extension period of DNA at 72°C for 10 min and the amplified products were sequenced.

The severity of symptoms produced by each unmodified/mutant virus was recorded 30 days after inoculation. The symptom severity on the foliage of SMV infected plants were recorded using a scale of 1 to 3, where 1 = mosaic on foliage; 2 = mosaic and rugosity; 3 = mosaic, rugosity with reduction in leaf lamina and leaf curling and severe stunting (Fig. 2.6) The relative accumulation of virus RNAs were quantified in plants infected with mutant viruses and compared to the average accumulation in SMV 413 and SMV G2 using quantitative real-time (q)RT-PCR (Power SYBR Green RNA-to-CT 1-Step Kit, Applied Biosystems Inc, Foster City, CA) with soybean actin as the internal control (Livak and Schmittgen, 2001). The qRT-PCR was carried out with SMV real-time universal primers SMV4618F (5'-CWACYCTTGACACAGAYAACCG-3') and SMV4742R (5'-TTCCTCAAYYCCTTTTCCYG-3') and specific primers for soybean actin gene Act1289F (5'-CCTGATGGGCAGGTTATCACT-3') and Act1349R (5'-GGTACAAGACCTCCGGACAC-3'). The thermal cycling conditions for the real-time RT-PCR were 48°C for 30 min for reverse transcription and 95°C for 10 min for activation of the polymerase followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation curve was generated at the end of the cycle to detect non-specific amplification (95°C for 15 s, 60°C for 15 s and 95°C for 15 s).

Mature seeds were harvested from SMV-infected plants and seed transmission rates were determined using grow-out tests by planting 108 seeds (or fewer depending on the number of seeds produced) in 72-well polystyrene trays containing soilless mix (Sunshine Mix LC1; Sun

Gro Horticulture Inc., Bellevue, WA). SMV infection of seedlings was determined by visual inspection. The experiment was conducted in three replications in the greenhouse at 25°C to 27°C with a 12 h photoperiod. The statistical analysis to compare the average germination percentage and seed transmission rates of the modified/unmodified SMV was computed using generalized linear model assuming a binomial distribution with a logit link function in PROC GLIMMIX in SAS. A constant value of 0.375 was added to every data point to eliminate zeros in the data set as zeros lead to inflated standard error of means.

Aphid transmission

The aphid transmissibilities of the recombinant viruses were conducted on soybean ‘Williams 82’ with apterous aphids, *A. glycines*, that were maintained in controlled environment chambers at 24°C on SMV-free ‘Williams 82’ plants. Aphids were collected and starved for 30 min and given an acquisition access feed (AAF) of 2 min on infected leaves from each of the unmodified/mutant SMV clones. Accumulation of SMV RNA in the infected leaf tissue of ‘Williams 82’ used in AAF was quantified using quantitative real-time RT-PCR as described earlier. Ten unifoliate seedlings were inoculated with each of the 15 unmodified/mutant SMVs by transferring five aphids/plant for an inoculation access feed (IAF) of 24 h. The aphid infested plants during IAF were placed in cages to avoid aphid movement among plants. The plants were then fumigated for 2 h with Hot Shot No-Pest Strip (Chemsico, St. Louis, MO) in a controlled environment chamber and then maintained in the greenhouse for three weeks for symptom expression. The plants were assayed for SMV by TBIA or by sequencing of RT-PCR products as described earlier. Aphid transmission rates were compared using a generalized linear model

assuming a binomial distribution with a logit link function in PROC GLIMMIX in SAS with a constant value of 0.375 added to every data point.

Sequence analysis

Infectious clones of SMV 413 (GenBank accession number GU015011) and SMV G2 were sequenced using primers spaced 1 kb apart (Table 2.1) as described earlier. Primers were designed using Primer3 (Rozen and Skaletsky, 2000). Sequencher 4.7 (Gene Codes, Ann Arbor, MI) and DNAMAN (Lynnon BioSoft, Quebec, Canada) were used for sequence assembly, amino acid sequences predictions and sequence alignments.

RESULTS

Symptom development

Unmodified SMV 413, SMV G2 and the mutant viruses showed varying levels of symptom severity on the vegetative tissue of 'PI 68671' (Table 2.2). The SMV 413 and SMV G2 shared 96.93% identity in their predicted amino acid sequences and shared symptoms such as stunting and foliar symptoms of mosaic, rugosity and leaf curling. The mutants p4G-Sal, p4G-Sal-CP, pG2-DM and pG2-HPmut produced symptoms that were less severe than either SMV 413 or SMV G2. The mutants p413-CP1mut, p413-CP2mut, p413-CP-DM, p413-NIbmut, p413-P1mut and pG2-CPmut produced symptoms with severities similar to those induced by SMV 413 and SMV G2. However clones pG4-Sal, p413-HPmut and p413-DM produced symptoms much more

severe than those produced by SMV 413 or SMV G2. Replacing the HC-Pro coding region in SMV 413 with that from SMV G2 was associated with increased symptom severity and replacing the HC-Pro coding region of SMV G2 with that of SMV 413 was associated with decreased symptom severity. The variability of the symptoms induced by the clones were not the result of differential levels of SMV RNA accumulation in the infected tissues as p413-HPmut and p413DM with the most severe symptoms had highest and the lowest SMV accumulation relative to the average accumulation in unmodified SMV. Also mutants that produced less severe symptoms did not differ significantly in viral RNA accumulation at 30 days post inoculation (Table 2.3).

Seed coat mottling

The soybean seeds from 'PI 68671' infected with unmodified SMV 413 and SMV G2 and mutant viruses exhibited differential mottling of soybean seed coats (Fig. 2.7) Seeds from plants of soybean 'PI 68671' infected with SMV 413 showed seed coat mottling whereas seeds from plants infected with SMV G2 were free from seed coat mottling (Table 2.2). Mutants p413-CP1mut, p413-NIbmut, p413-P1mut, p413-HPmut, p413-DM, and pG4-Sal showed extensive pigmentation on the seed coat. Mutants p413-CP2mut, p413-CP-DM, pG2-CPmut, pG2-HPmut, pG2-DM, p4G-Sal and p4G-Sal-CP lacked seed coat mottling. The results indicate that the loss of SMV-induced seed coat mottling in soybean 'PI 68671' was linked to a single amino acid residue change from Q to P at position 264 in the C-terminus of the CP (Fig. 2.4)

Seed transmission

The seed transmission rate of SMV in 'PI 68671' was influenced by multiple protein coding regions and specific amino acid residues in the SMV genome (F -test was significant at $P = <0.0001$). The unmodified SMV G2 lacked seed transmission and the SMV 413 was seed transmitted in 14% of the seedlings. The unmodified strain, SMV 413 and the mutant p413-P1mut were the only seed transmitted strains. p413-P1, a SMV 413 with a P1 gene inserted from SMV G2, had the highest seed transmission rate of 26% which was significantly higher ($P = 0.002$) than SMV 413 (Fig. 2.8). All the other mutants lacked seed transmission or had very low seed transmission which was not significantly different, at $P < 0.05$, from the non-seed transmitted SMV G2 (Fig. 2.8). The mutant pG4-Sal had very low seed set due to extremely severe virus symptoms and was not included in the grow-out tests to determine seed transmission.

The P1 coding region of SMV G2 increased seed transmission rates in SMV 413. The substitution of the HC-Pro coding region of SMV 413 in p413-HPmut and p413-DM with that of SMV G2 resulted in the loss of seed transmission. Replacement of HC-Pro in SMV G2 in pG2-HPmut or the restoration of the amino acid motif DAG (Fig. 2.3) at the N-terminus in the CP in pG2-CPmut or both the mutations together in pG2-DM did not affect the seed transmission rates which remained at 0% similar to the SMV G2. The lack of seed transmission of p4G-Sal and p4G-Sal-CP indicated that regions in CP, CI, NIa, NIb or VpG may influence seed transmission excluding the 6K protein that had 100% amino acid sequence identity between the unmodified SMV isolates. Mutations to SMV 413 amino acid residue G12 in the amino acid motif DAG of

the CP in p413-CP1mut and the amino acid residue Q264 at the C-terminus (Fig 2.4) of CP in p413-CP2mut lead to the loss of seed transmission implicating both the amino acids in seed transmission. The loss of seed transmission in 413-NIbmut indicates that NIb protein from SMV G2 with D instead of G in the amino acid motif DAG in the CP can eliminate seed transmission in SMV 413.

The difference of the seed transmission levels were not influenced by virus accumulation in the 'PI 68671'. Accumulation of SMV genomic RNA in the seed-transmitted strains SMV 413 and p413-P1mut were not significantly different from the non-seed-transmitted strains (Table 2.3). Differences in seed transmission rates were not influenced by the percent seed germination as the mutant strains, with the exception of p413-CP1 and p413-DM, did not differ significantly ($P < 0.05$) in the seed germination rates from unmodified SMV isolates. p413-CP1 and p413-DM had very low seed transmission compared to unmodified SMV 413.

Aphid transmission

Transmission of SMV by *A. glycines* in soybean 'Williams 82' was dependent on the SMV isolate (F -value significant at $P < 0.0001$), with p413-CP2mut transmitted at the highest transmission rate of 63% (Fig. 2.9). The aphid transmission rates of SMV 413, pG2-DM, p4G-Sal-CP, p413-P1mut were not significantly different ($P < 0.05$) from the highest transmission rate recorded in p413-CP2mut. SMV G2, pG4-Sal, pG2-HPmut, p4G-Sal, p413-NIbmut, p413-DM, p413-CP-DM and p413-CP1mut were not aphid transmitted whereas transmission of pG2-CPmut was recorded in very few plants and was not significantly different from non-aphid

transmitted unmodified SMV G2 ($P = 0.50$) or the mutants. The mutant p413-HPmut had a low aphid transmission which was not significantly different from pG2-DM ($P = 0.05$, aphid transmitted) or SMV G2 ($P = 0.21$, non-aphid transmitted) (Fig. 2.9).

The aphid transmissibility of SMV G2 was restored in pG2-DM by the introduction of HC-Pro from SMV 413 and the DAG motif in the CP (Figs. 2.3, 2.5). Loss of the DAG motif from the unmodified SMV 413 in p413-CP1mut abrogated aphid transmission. The mutant p4G-Sal-CP, with 5' half of the virus derived from SMV 413 and the 3' half of the virus derived from SMV G2 and the DAG motif in the CP restored, was efficiently aphid transmitted whereas p4G-Sal lacked aphid transmission. These results illustrate the importance of the DAG motif of the coat protein in aphid transmission. However, the substitution of Q to P at amino acid position 264 near the C terminus of the CP of SMV 413 in p413-CP2mut did not affect aphid transmission (Figs. 2.4, 2.5)

The HC-Pro coding region played a secondary role in aphid transmission, which was evident by the reduction of transmission with the substitution of HC-Pro in SMV 413 with HC-Pro from SMV G2 in p413-HPmut. The amino acid sequences of HC-Pros of SMV 413 and SMV G2 are 95.6% identical, but the first 100 amino acid residues that are exclusively involved in aphid transmission (Dolja et al., 1993) contains most of the mismatches with 88% identity (Fig. 2.10). Amino acid residues that have been previously linked to aphid transmission were conserved between the SMV 413 and SMV G2 (Flasinski and Cassidy, 1998; Seo et al., 2010). SMV protein P1, which is the most variable protein between the unmodified SMVs (88.6% identity) did not influence the transmission rates as evident from efficient aphid transmission of p413-

P1mut. pG2-CPmut had the highest accumulation of SMV genomic RNA followed by p4G-Sal, p413-CP-DM and p413-CP1 which were not aphid transmitted (Table 2.4). The levels of SMV RNA accumulation in the aphid transmitted clones SMV 413, p413-CP2mut, p413-P1mut, pG2-DM and p4G-Sal-CP were lower than most of the non-transmitted clones with the lowest accumulation recorded in p413-NIbmut (not aphid transmitted).

DISCUSSION

In this study, unmodified isolates SMV 413 and SMV G2 and mutants engineered from the two isolates were compared to investigate the role of SMV proteins and specific amino acid residues in foliar symptom severity, seed coat mottling, seed transmission and aphid transmission. The HC-Pro region was associated with induction of severe symptoms by SMV in soybean, 'PI 68671'. HC-Pro has been previously reported to be involved in SMV and ZYMV (potyvirus), in symptom development (Desbiez et al., 2010; Lim et al., 2007). In ZYMV, the amino acid motif CDNQLD is responsible for symptom severity (Desbiez et al., 2010) however, the motif was conserved in the SMV isolates, SMV 413 and SMV G2. HC-Pro is a suppressor of gene silencing (Ebhardt et al., 2005; Merai et al., 2006; Moissiad et al., 2011), suppressors of gene silencing can affect symptom severity (Lim et al., 2007), but P1, also linked to suppression of gene silencing (Giner et al., 2010), had no effect on the symptom severity of the virus isolates tested in 'PI 68671'.

The difference in levels of seed coat mottling between SMV 413 and SMV G2 in 'PI 68671' was linked to a single amino acid residue Q264 near the C-terminus of CP. As has been previously

reported, the presence of seed coat mottling is not a good indicator of seed transmission (Domier et al., 2007; Pacumbaba, 1995), and both seed transmitted and non-seed transmitted SMV isolates exhibited seed coat mottling in 'PI 68671'. Previous studies found that the extent of seed coat mottling and seed transmission are dependent on the interactions between soybean cultivar and SMV isolate (Domier et al., 2007; Hobbs et al., 2003; Porto and Hagedorn, 1975; To, 1989) and the soybean growth stage at the time of infection (Ren et al., 1996). In 'PI 68671', seed transmission of SMV is controlled by multiple regions within the SMV genome. P1, an RNA silencing suppressor, affected seed transmission by increasing the transmission rates of unmodified SMV 413. This result is consistent with the association of chromosomal regions in the soybean genome with genes involved in induction of viral RNA silencing with seed transmission of SMV (Domier et al., 2011). HC-Pro, a RNA silencing suppressor, affected seed transmission evidenced by the inhibition of seed transmission in SMV 413 mutants that had HC-Pro coding region from SMV G2. Johansen et al. (1996) also observed the importance of the HC-Pro coding region of PSbMV in seed transmission. These results point to the role of RNA silencing in SMV seed transmission in soybean. Single amino acid changes in the CP of SMV 413 of G12 to D in the DAG motif and Q264 to P near the C-terminus of CP abolished SMV seed transmission in 'PI 68671'. The role of DAG motif is required for aphid transmission of multiple potyviruses and facilitates interactions between SMV HC-Pro and CP (Seo et al., 2010), but it has not been reported earlier from SMV or any potyviruses to be a determinant of seed transmission. The influence of the DAG motif and the C-terminus of the CP, which are associated with HC-Pro and CP interactions (Seo et al., 2010), indicates that such interactions might also be required for seed transmission.

The aphid transmission of SMV was influenced primarily by the amino acid G12 in the DAG motif in the CP and HC-Pro played a secondary role. This result is a further confirmation of the importance of the DAG motif and HC-Pro in aphid transmission that has been reported in SMV and other potyviruses (Atreya et al., 1990; Blanc et al., 1997; Blanc et al., 1998; Seo et al., 2010). The N-terminus of the HC-Pro harbors most of the amino acid mismatches between HC-Pro of SMV 413 and SMV G2 implicating this region in aphid transmission as has been reported previously (Dolja et al., 1993). The role of DAG motif of CP and HC-Pro was also evident by the restoration of aphid transmission in the non-transmitted SMV G2 with the mutation of D12 to G in the DAG motif and by replacing the SMV G2 HC-Pro with SMV 413 HC-Pro. Further studies will be required to identify specific regions or amino acid within P1, HC-Pro and N1b that can influence seed transmission.

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TABLES

Table 2.1. List of primers used for sequencing *Soybean mosaic virus* (SMV) 413, SMV G2 and construction of recombinant viruses.

Primers	Nucleotide sequence 5'→3'
540R-SMVG2	CGGAAACACCAGCGTAAAAT
558F-SMVG2	TTTGAAGGAGGGAGTGTTGG
558F-SMVG2	TTTGAAGGAGGGAGTGTTGG
818R-SMV	TTGACGTTCCCTGCCTTTACC
964F-SMV	TGAGCGATCATCTTTAACCACA
1327F-SMVG2	AAGAAATTGACGCGCATGAGG
1676R-SMV	TGGAAGATCGCTTATTCCTGA
1813R-SMVG2	AGTGACCCAATTGCCAACTC
1820F-SMV	CCCTCTGAAGGGTACAGCAA
1888F-SMV	GGGAATTAGCGATTGGGTCT
2180F-SMVG2	GTTAGGAAAGTGGCCGACAA
2832R-SMVG2	CCAATTGGTTGTTGGATGCT
2856F-SMV	CGCCACAGTCATACAAGACG
3565F-SMV	GGCCAAAACAGCAACTCAAT
3592R-SMV	TGAATGCCACGATTTTCTCA
4133F-SMV	TGAGAGGGTTGAGGCAAGTT
4178R-SMV	GAAACGCAAAACCCACTTGT
4259F-SMVG2	CGAATTCACAACGCAACATC
4735F-SMV	CGGTGTGTGCGTTACAACA
4890R-SMV	GTACTTTATGAAGTTAGTGGTGAAAAATGGAGTTAG
4929F-SMVG2	GGTAGCATGCACCCAGAAAT
5849R-SMV	CGTACACCTCACGGCCTACT
5849F-SMV	AGTAGGCCCGTGAGGTGTACG
6131F-SMVG2	TCTCTGCCAAAACAGCAATG
6551F-SMV	TGGCATGGTGAGTTTGTGAT
6572R-SMV	TTGAGCTGTGTGGTGTGTGTG
6869R-SMV	TCAGTGAGTGGGACGAAGAA
7146F-SMV	CAAAGCACGTTGTGAAAGGA
7230R-SMVG2	CTGCCCTCTCAAAAGATTGG
7527F-SMVG2	AGTGTGGAATGGATCCCTGA
7573R-SMVG2	GTGTCAATTGGTGCTGCTGT
7824R-SMV	ACCCATCCATCGGGTAAACT
8238F-SMV	CTAGAGTGGGACAGGAGCAAAG
8268F-SMVG2	CCCACAAAGCCGTGTTAGTT
8625R-SMV	CCAGCTCCTTTGCTACTGCT
8662F-SMVG2	CGCGTTTGCGAAGATTACA
8919F-SMV	ATGATGAGCAGATGGGTGTG
9406F-SMV	CTGCAAGGGATGTGAATCAA
9455R-SMV	TCACACTATACTCAAAGAAAGTGCAA
14245F-SMVG2	CGCACAATCCCCTATCCTT
14273R-SMVG2	GGGTCTTGCGAAGGATAGTG

Table 2.2. Symptom severity 30 days after inoculation and the seed coat mottling on mature seeds of ‘PI 68671’ infected by *Soybean mosaic virus* (SMV) unmodified and mutants.

SMV clones	Symptom severity ^a	Seed coat mottling ^b
p4G-Sal	1	Unmottled
p4G-Sal-CP	1	Unmottled
pG2-DM	1	Unmottled
pG2-HPmut	1	Unmottled
SMV 413	2	Mottled
SMV G2	2	Unmottled
p413-CP1mut	2	Mottled
p413-CP2mut	2	Unmottled
p413-CP-DM	2	Unmottled
p413-NIbmut	2	Mottled
p413-P1mut	2	Mottled
pG2-CPmut	2	Unmottled
pG4-Sal	3	Mottled
p413-HPmut	3	Mottled
p413-DM	3	Mottled

^a Symptom severity score: 1 = mosaic on foliage; 2 = mosaic and rugosity; 3 = mosaic, rugosity with reduction in leaf lamina and leaf curling and severe stunting.

^b Seed coat mottling: Unmottled = yellow seed; Mottled = seed with brown pigmentation.

Table 2.3. The comparison of the *Soybean mosaic virus* (SMV) RNA accumulated in infected tissue of ‘PI 68671’ (30 day post inoculation) in unmodified/mutant SMV with the average of accumulation in SMV 413 and SMV G2.

SMV clone	ΔCt	$\Delta\Delta Ct$	$2^{-\Delta\Delta Ct}$
SMV-G2	-6.7 ± 0.2^a	0.1 ± 0.2^b	$0.9 (0.8 - 1)^c$
SMV-413	-6.9 ± 0.4	-0.1 ± 0.4	$1.1 (0.8 - 1.4)$
pG4-Sal	-6 ± 0.3	0.8 ± 0.3	$0.6 (0.5 - 0.7)$
pG2-HPmut	-6 ± 0.3	0.8 ± 0.3	$0.6 (0.5 - 0.7)$
pG2-DM	-5.9 ± 0.1	0.9 ± 0.1	$0.5 (0.5 - 0.6)$
pG2-CPmut	-7.2 ± 0.4	-0.4 ± 0.4	$1.3 (1 - 1.7)$
p4G-Sal-CP	-6.2 ± 0.3	0.6 ± 0.3	$0.7 (0.5 - 0.8)$
p4G-Sal	-6.8 ± 0.3	0 ± 0.3	$1 (0.8 - 1.2)$
p413-P1mut	-6.3 ± 0.4	0.5 ± 0.4	$0.7 (0.6 - 0.9)$
p413-NIbmut	-6.4 ± 0.3	0.4 ± 0.3	$0.8 (0.6 - 0.9)$
p413-HPmut	-7.7 ± 0.2	-0.9 ± 0.2	$1.9 (1.6 - 2.1)$
p413-DM	-4.9 ± 0.2	1.9 ± 0.2	$0.3 (0.2 - 0.3)$
p413-CP-DM	-6.8 ± 0.1	0 ± 0.1	$1.0 (1 - 1)$
p413-CP2mut	-6.1 ± 0.4	0.7 ± 0.4	$0.6 (0.5 - 0.8)$
p413-CP1mut	-7.3 ± 0.3	-0.5 ± 0.3	$1.4 (1.1 - 1.8)$

^a ΔCt (SMV G2 RNA levels normalized to soybean actin gene expression) = Mean of SMV G2 Ct_{SMV} - Mean of SMV G2 Ct_{Actin}

^b $\Delta\Delta Ct$ (relative expression of SMV genomic RNA in SMV G2- Average of SMV G2 and SMV413) = ΔCt_{G2} - $\Delta Ct_{Average\ of\ SMV\ G2/413}$

^c $2^{-\Delta\Delta Ct}$ is folds or the relative amount SMV genomic RNA in SMV G2 to average of SMV G2 and SMV413.

Table 2.4. The relative accumulation of *Soybean mosaic virus* (SMV) RNA in ‘Williams 82’ infected with unmodified/mutant SMV with the average SMV accumulation recorded in SMV 413 and SMV G2, 30 days post inoculation.

SMV clone	ΔCt	$\Delta\Delta Ct$	$2^{-\Delta\Delta Ct}$
SMV-G2	-7.6 ± 0.1	-0.4 ± 0.1	1.3 (1.4 ± 1.2)
SMV-413	-6.7 ± 0.2	0.4 ± 0.2	0.8 (0.9 ± 0.7)
pG4-Sal	-7.8 ± 0.5	-0.6 ± 0.5	1.5 (2.1 ± 1.1)
pG2-HPmut	-6.5 ± 0.3	0.7 ± 0.3	0.6 (0.8 ± 0.5)
pG2-DM	-6.5 ± 0.2	0.7 ± 0.2	0.6 (0.7 ± 0.5)
pG2-CPmut	-8.8 ± 0.2	-1.6 ± 0.2	3 (3.5 ± 2.6)
p4G-Sal-CP	-7.1 ± 0.5	0.1 ± 0.5	0.9 (1.3 ± 0.7)
p4G-Sal	-8.1 ± 0.3	-1 ± 0.3	2 (2.5 ± 1.6)
p413-P1mut	-6.7 ± 0.1	0.5 ± 0.1	0.7 (0.8 ± 0.7)
p413-NIbmut	-6.1 ± 0.3	1.1 ± 0.3	0.5 (0.6 ± 0.4)
p413-HPmut	-7.4 ± 0.7	-0.3 ± 0.7	1.2 (2 ± 0.8)
p413-DM	-7.8 ± 0.1	-0.7 ± 0.1	1.6 (1.7 ± 1.5)
p413-CP-DM	-8.1 ± 0.4	-1 ± 0.4	2 (2.6 ± 1.5)
p413-CP2mut	-7 ± 0.2	0.2 ± 0.2	0.9 (1 ± 0.8)
p413-CP1mut	-8.1 ± 0.1	-1 ± 0.1	2 (2.1 ± 1.9)

^a ΔCt (SMV G2 RNA levels normalized to soybean actin gene expression) = Mean of SMV G2 Ct_{SMV} - Mean of SMV G2 Ct_{Actin}

^b $\Delta\Delta Ct$ (relative expression of SMV genomic RNA in SMV G2- Average of SMV G2 and SMV413) = ΔCt_{G2} - $\Delta Ct_{Average\ of\ SMV\ G2/413}$

^c $2^{-\Delta\Delta Ct}$ is folds or the relative amount SMV genomic RNA in SMV G2 to average of SMV G2 and SMV413.

FIGURES

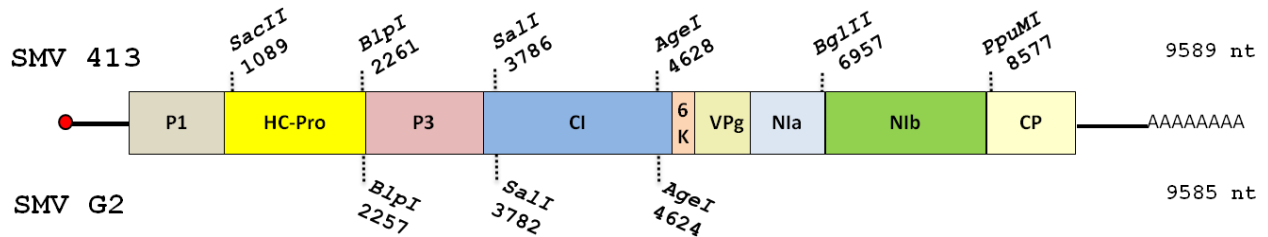


Figure 2.1. The restriction sites within polyprotein of the full-length *Soybean mosaic virus* (SMV) isolates SMV 413 and SMV G2 used in construction of chimeric SMV. The proteolytic cleavage sites for P1, helper component proteinase (HC-Pro), P3, cytoplasmic inclusion (CI), 6K, viral genomic protein (VPg), nuclear inclusion a (NIa), nuclear inclusion b (NIb) and coat protein (CP) coding regions are indicated. The nucleotide positions of the restriction sites used in the development of chimeric SMV are shown for SMV413 (above) and SMV G2 (below) the cleavage map.

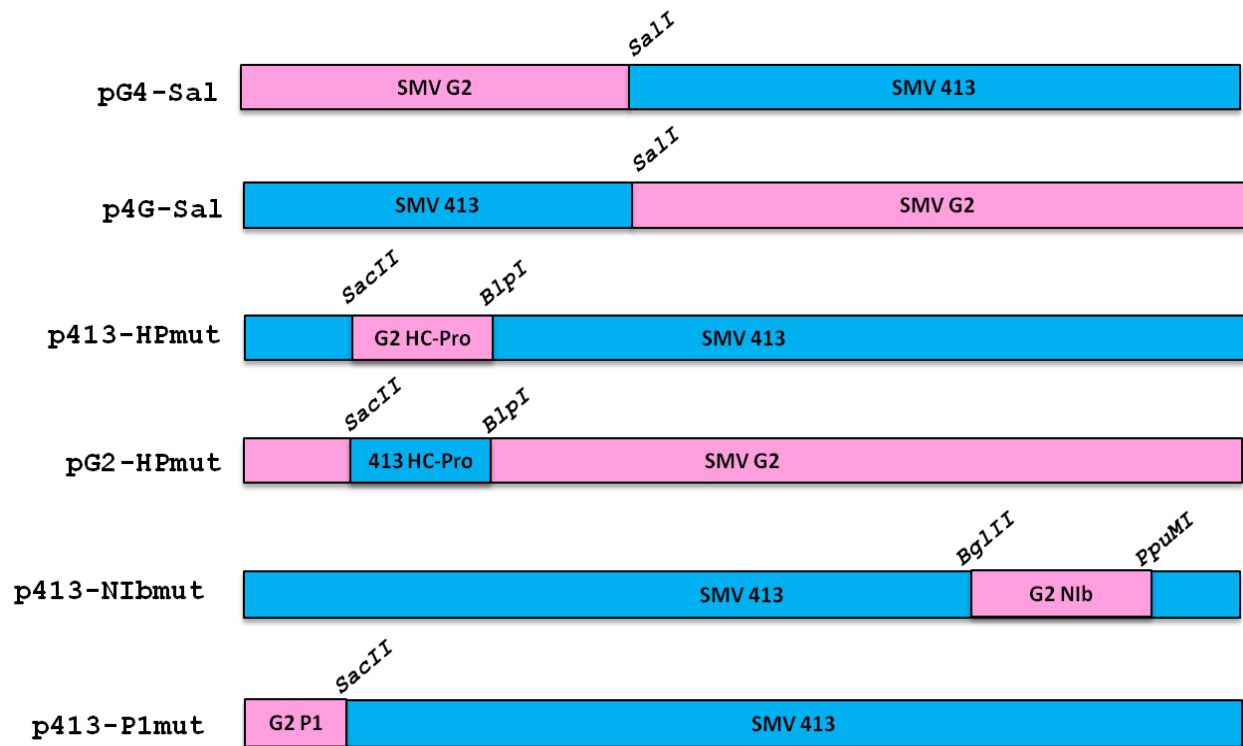


Figure 2.2. Mutant *Soybean mosaic virus* (SMV) clones pG4-Sal and p4G-Sal constructed using the unique *SalI* site present in both the unmodified viruses. The clones p413-HPmut and pG2-HPmut were developed using *SacII* and *BlnI* near the proteolytic cleavage site of the helper component protease (HC-Pro). Mutant p413-NIbmut was made using the *BglII* and *PpuMI* sites to insert the coding region of nuclear inclusion b (NIb) regions from SMV G2 in SMV 413 and the *SacII* site to insert SMV G2 P1 coding region into SMV 413.

(A)

		1		35-		60
SMV 413		TCAGGCAAGGAGAAAGAAGGAGACATGGATGCAG	GC	CAAGGACCCAAAGAAGAGTACCAGC		
SMV G2		TCAGGCAAGGAGAAGGAAGGAGATATGGATGCAG	ATA	AAGGATCCAAAGAAGAGCACCAGT		

(B)

		1		20
SMV 413		SGKEKEGDMD	AG	KDPKKSTS
SMV G2		SGKEKEGDMD	AD	KDPKKSTS

Figure 2.3. The nucleotide and amino acid sequence mismatches near the N-termini in coat proteins (CPs) of *Soybean mosaic virus* (SMV) isolates SMV 413 and SMV G2. (A) The 60 nucleotides (nt) from the 5' terminus of the CP coding regions of SMV 413 and SMV G2 with the nts in red at position 35 and 36 that produce the amino acid change of G in SMV 413 to D in SMV G2. The other nt changes are translationally silent. (B) The 20 predicted amino acid from the N-termini of the coat proteins of SMV 413 and SMV G2 with the DAG motif highlighted, which is present in SMV 413, but modified in SMV G2.

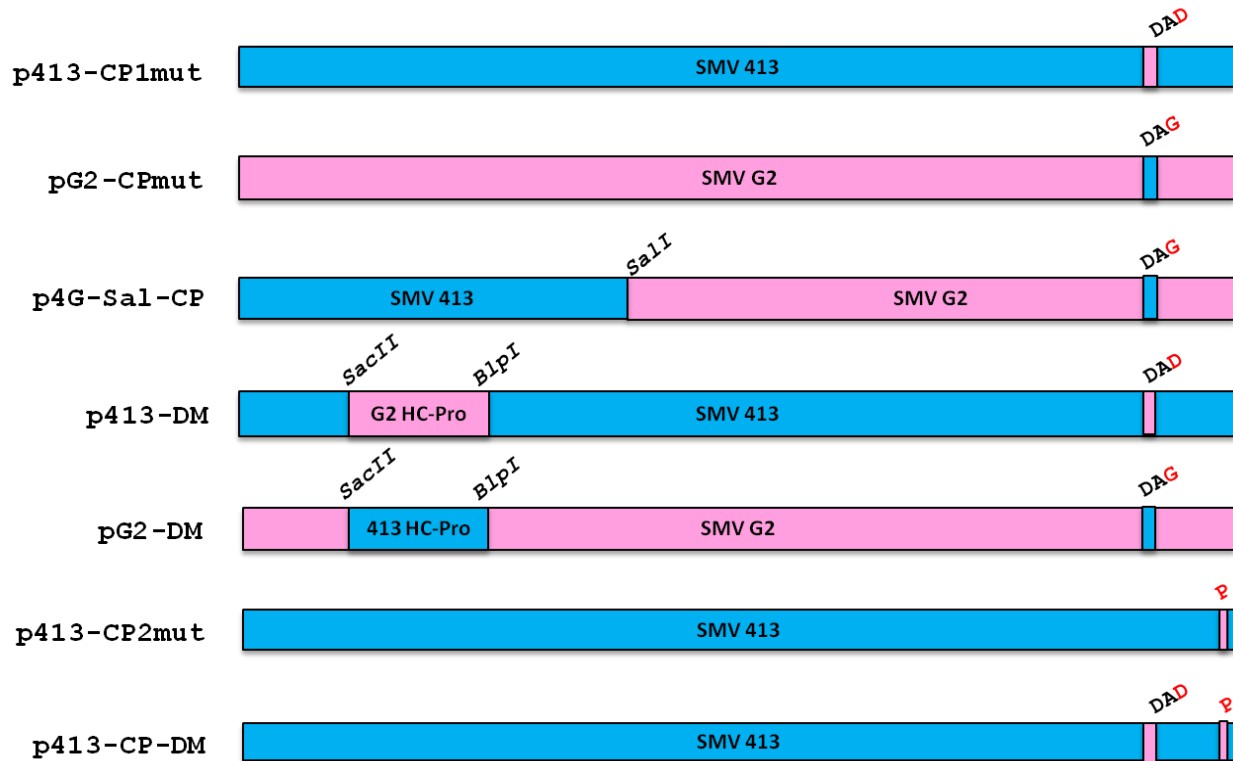


Figure 2.5. Mutant *Soybean mosaic virus* (SMV) clones, p413-CP1mut with single amino acid substitution of G12 with D in the DAG motif of the coat protein (CP) of SMV 413, pG2-CPmut with a single amino acid substitution to restore the DAG motif in CP of SMV G2, p4G-Sal-CP has the DAG motif restored in the clone p4G-Sal, p413-DM has the helper component protease (HC-Pro) of SMV G2 in SMV 413 and has a single amino acid substitution of G to D in the DAG motif of the CP, pG2-DM has the HC-Pro of SMV G2 replaced by that of SMV 413 and the DAG motif inserted in the SMV G2 CP, p413-CP2mut has a single mutation of Q264 to P near the C-terminus of the CP of SMV 413, p413-CP-DM has two mutation in the CP of SMV 413 of the DAG to DAD at the N-terminus and Q264 to P near the C-terminus.

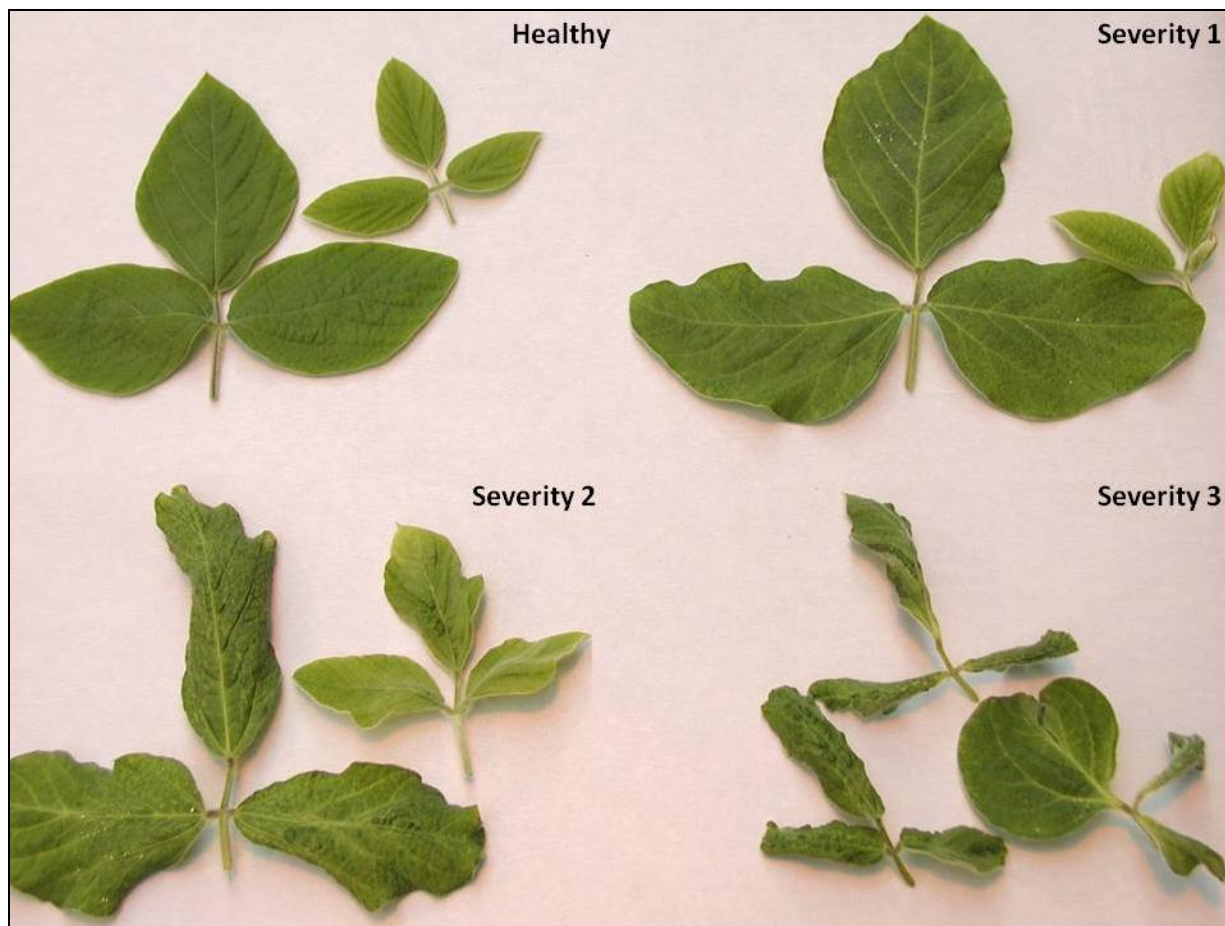


Figure 2.6. Foliage from healthy and *Soybean mosaic virus* (SMV)-infected soybean ‘PI 68671’ exhibiting symptoms at varying rates of severity from scale 1 to 3 as indicated, 30 days after mechanical inoculation at unifoliate stage with SMV (1: p4G-Sal, 2: SMV 413 and 3: p413-DM) isolates.

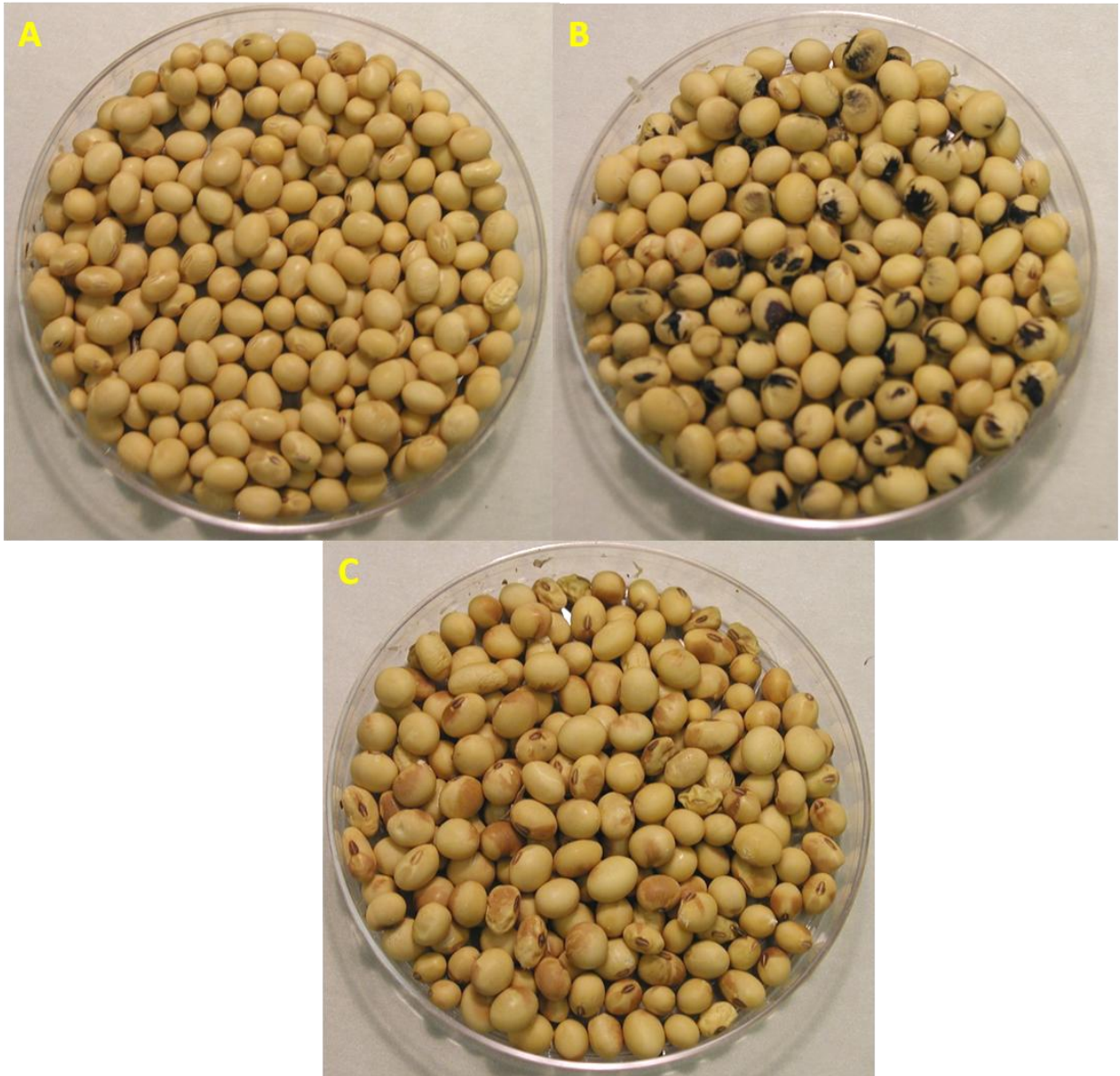


Figure 2.7. Seed coat pigmentation observed in seeds collected from soybean ‘PI 68671’ infected with A. SMV G2, B. p413-Nibmut and C. SMV 413.

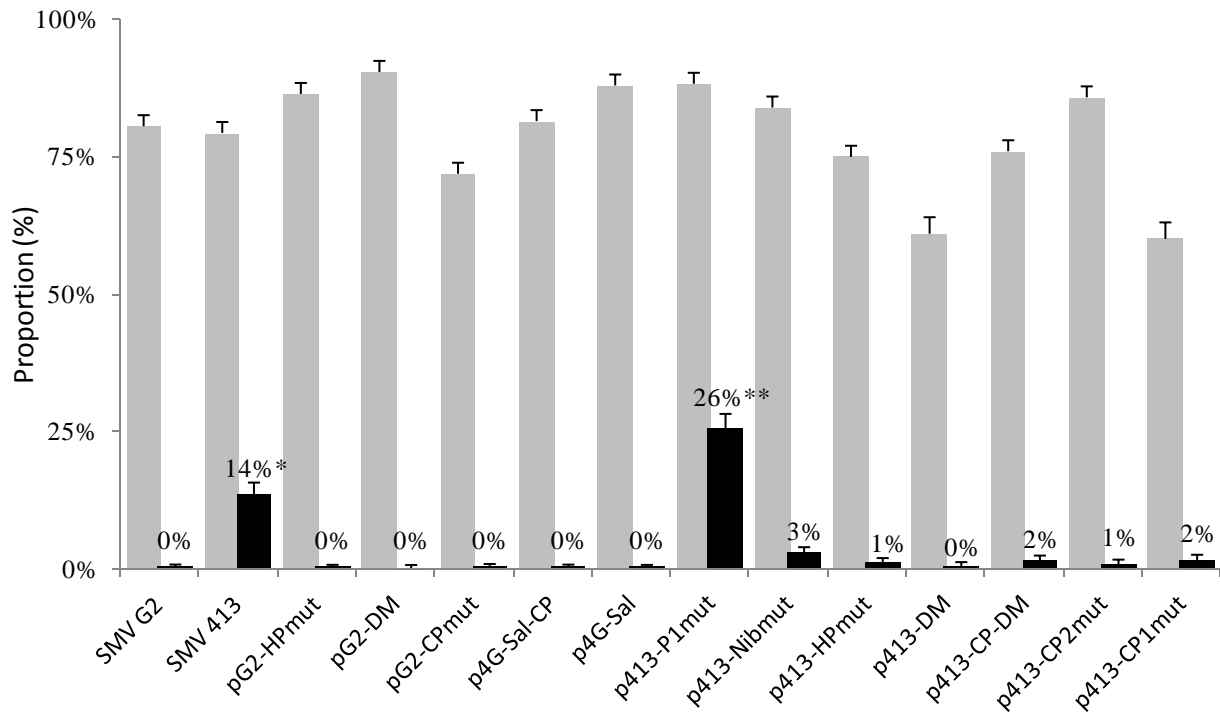


Figure 2.8. Mean germination percent of seeds in grow-out test (gray bars) and mean percent of seedling infected (black bars) with *Soybean mosaic virus* (SMV) by seed transmission of SMV G2, SMV 413 and recombinants in soybean ‘PI 68671’. The * indicates statistically significant difference at $P < 0.05$ in seed transmission and the error bars indicating the standard error of means.

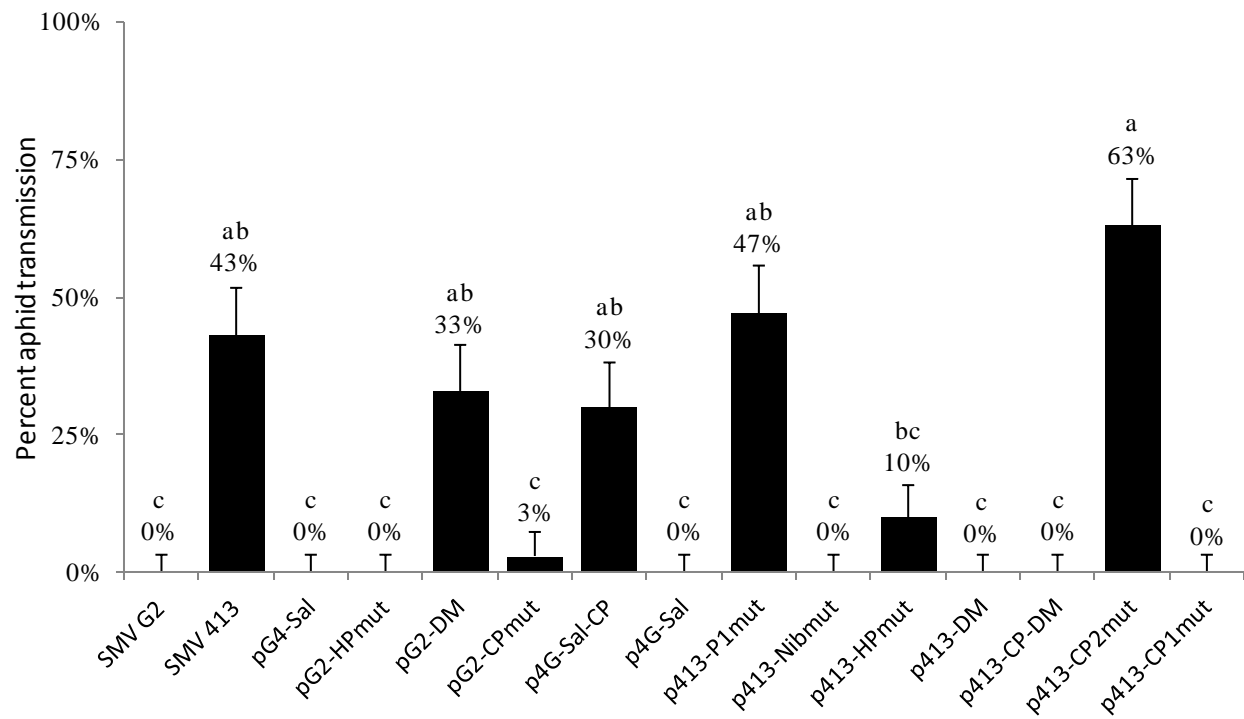


Figure 2.9. Percent aphid transmission by *Aphis glycines* of *Soybean mosaic virus* (SMV) isolates SMV G2, SMV 413 and mutants in soybean ‘Williams 82’ with the mean of percent aphid transmission and same letter (top of the bars) indicates that the transmission was not significantly different (based on confidence interval at $\alpha = 0.05$). The error bars indicate the standard error of means.

SMV_413_HC-Pro	SQTPEAQFFRGWKKVFDKMPPHVENHECTIDFTNEQCGEL	40
SMV_G2_HC-Pro	SQnPEAQFFRGWKKVFDKMPPHVENHECTtDFTNEQCGEL	40
Consensus	sq peaqqffrgwkkvfdkmpphvenhect dftneqcgel	
SMV_413_HC-Pro	AAAIQSVPVKK KLSC KQCRQHIK N LSWEEYKQFLLAHMG	80
SMV_G2_HC-Pro	AAAIQS <i>S</i> iFPVKK KLSC KQCRQHIK h LSWEEYKQFLLAHMG	80
Consensus	aaaisqs fpvk klsc kqcrq h ik lsweeykqfllahmg	
SMV_413_HC-Pro	CHRAEWENIQKVDGMKYVKKVIETSTAENASLQTSMEIVR	120
SMV_G2_HC-Pro	CHgpEWETfQeiDGMrYVKrVIETSTAENASLQTSleIVR	120
Consensus	ch ewe q dgm yvk vietstaenaslqts eivr	

Figure 2.10. The predicted amino acid sequence alignment of 120 amino acid residues from the N-terminus of the helper component protease (HC-Pro) of *Soybean mosaic virus* (SMV) isolates SMV 413 and SMV G2 along with the consensus sequence and the KLSC amino acid conserved motif indicated in the box.

CHAPTER 3

CONSTRUCTION OF A DNA-BASED VIRUS-INDUCED GENE SILENCING SYSTEM FOR FUNCTIONAL GENOMICS OF SOYBEAN SEED DEVELOPMENT

ABSTRACT

Virus induced gene silencing (VIGS) systems are functional genomics tools that permit transient knock down of gene expression. *Tobacco streak virus* (TSV), a seed transmitted virus in soybean, has a tripartite, single-stranded, positive-sense, RNA genome. TSV was developed into a DNA-based VIGS vector for investigating genes expressed in soybean seed. TSV RNAs 1, 2, 3, and 4 were cloned into pHST40 downstream of a *Cauliflower mosaic virus* 35S promoter. The TSV-based VIGS system was developed by designing and inserting multicloning sites (MCSs) into RNA1, RNA2 and RNA3 to facilitate insertion of soybean gene fragments. The MCSs introduced into two truncated 2b genes of TSV in RNA2 (pHR2bM and pHR2bS) and the 3' noncoding region of RNA1 (pHR1) were stably replicated in soybean. Fragments of 105 to 175 nucleotide (nt) from the soybean genes encoding phytoene desaturase and magnesium chelatase subunit H were maintained stable only in the TSV RNA2 -based vectors, pHR2bM and pHR2bS, in systemic leaves of inoculated soybean plants. The integrity of the inserted partial gene into VIGS vectors pHR2bM and pHR2bS were also maintained during seed transmission. Deep sequencing of small RNAs of TSV-infected plants revealed that small interfering (si)RNA (21-24-nt) were produced nonuniformly from both RNA strands of the virus with a bias for the sense strand. The 21-nt size siRNA was the most abundant, representing about 70 % of siRNAs. The

majority of hot spots of siRNA production, as indicated by high numbers of siRNA alignment to the TSV genome, were located in TSV RNA3 and to the movement protein coding region within RNA3.

INTRODUCTION

Plant viruses as transient vectors for heterologous gene expression are an attractive alternative to stably transformed plants mainly due to their small genome sizes, making them relatively easy to manipulate, and high replication levels, which can produce concomitantly high levels of foreign gene expression suitable for high-throughput applications (Lico et al., 2008; Scholthof et al., 1996). Virus expression vectors have been applied to the production of recombinant protein such as pharmaceutical proteins, commercial protein sweeteners and human anti-microbial proteins such as lysozyme (Lico et al., 2008). Virus-induced gene silencing (VIGS) is a related technology where virus vectors contain a fragment of a plant gene that is down regulated by post transcriptional gene silencing in plants infected with the chimeric virus. Hence, VIGS has become an efficient reverse genetics tool for plants such as soybean that have extensive genome duplication (Schlueter et al., 2004; Shoemaker et al., 1996; Stacey et al., 2004). The sequencing of the soybean genome (Schmutz et al., 2010) has greatly enhanced the ability to use VIGS an important tool to identify specific gene function (Jackson et al., 2006) and verify candidate gene effects (Meyer et al., 2009).

VIGS vectors exploit the plant defenses against viruses that are triggered by double stranded (ds) RNA present in the replicative-intermediates and/or secondary structures of virus RNA genomes

(Fusaro et al., 2006). RNA silencing is initiated by the binding of a RNase III-like enzyme, Dicer-like (DCL) that cleaves dsRNA into small interfering (si)RNA duplexes of 21-24 nucleotides (nt) (Fusaro et al., 2006). These siRNA duplexes can initiate and spread silencing in plants (Dunoyer et al., 2010). One strand from the siRNA duplex is loaded into Argonaute protein (AGO), an RNase H-like enzyme, forming the RNA-induced silencing complex (RISC), which can target complementary single-stranded (ss) RNA for cleavage or translation repression (Baumberger and Baulcombe, 2005; Brodersen et al., 2008; Qi et al., 2005). Plant RNA-dependent RNA polymerases (RDRs) amplify the silencing signals and induce the production of secondary siRNAs (Garcia-Ruiz et al., 2010; Qu et al., 2008; Wang et al., 2010).

In soybean (*Glycine max* (L.) Merr), a VIGS vector based on *Bean pod mottle virus* (BPMV), a bipartite comovirus, was developed by inserting plant genes between the movement protein (MP) and the coat protein (CP) in BPMV RNA2 (Zhang and Ghabrial 2006); and enhanced by cloning the BPMV cDNAs downstream of a *Cauliflower mosaic virus* (CaMV) 35S promoter (Zhang et al. 2009). The vector has been used to discern the role of soybean genes responsible in soybean rust resistance (Meyer et al., 2009) a mitogen-activated protein kinase in plant defense response (Liu et al., 2011; Zhang et al., 2009), ribosomal proteins in translation and actin in the cytoskeleton (Zhang et al., 2009). However, BPMV is not seed transmissible and does not invade meristems, and consequently cannot be used in studying genes in embryos or meristems (Krell et al., 2003). Seed transmitted VIGS vectors derived from *Cucumber mosaic virus* (CMV) (Nagamatsu et al., 2007) and *Apple latent spherical virus* (ASLV) (Yamagishi and Yoshikawa, 2009) have also been developed in soybean and utilized to examine genes involved in the flavonoid pathway in seeds and seedlings, but show relatively low levels of seed transmission in

soybean. In the CMV- and ASLV-based VIGS vectors, viral cDNAs are cloned downstream of prokaryotic promoters that require *in vitro* transcription for inoculation. DNA-based vectors that transcribe plant virus genomes *in vivo* from a CaMV 35S promoter are amenable to direct inoculation, eliminating the need for RNA transcription and making them more suited to high-throughput applications (Zhang et al., 2009).

Tobacco streak virus (TSV) is a single-stranded, positive-sense, tripartite RNA virus of the genus *Ilarvirus* and family *Bromoviridae* (Fig. 1.2) (Hull, 2002). The genomic RNAs have methylated 5' caps and several stem-loop structures with interspersed AUGC motifs in the 3' noncoding region (Hull, 2002; Reusken and Bol, 1996). RNA1 is 3.5 kb and encodes a single protein that includes methyltransferase and helicase motifs (Scott et al., 1998). RNA2 is 2.9 kb and encodes the 2a and 2b proteins from overlapping open reading frames (Scott et al., 1998). The 2a protein is an RNA-dependent RNA polymerase (Scott et al., 1998) and the 2b protein has been suggested to be associated with suppression of RNA silencing and virus movement by similarity to the 2b protein of CMV (Brigneti et al., 1998; Shi et al., 2003; Xin and Ding, 2003). RNA3 is 2.2 kb and encodes the MP and CP (Cornelissen et al., 1984). The CP is essential for TSV infection and expressed from a subgenomic RNA4, that is 3'-coterminial with RNA3 (Vloten-Doting, 1975).

TSV causes severe initial symptoms such as leaf puckering, darkening along petioles and veins, with crooked stem tips and bud blight (Almeida et al., 2005; Fagbenle and Ford, 1970; Ghanekar and Schwenk, 1974) followed by symptom recovery (Fulton, 1978; Xin and Ding, 2003). The recovery phenomenon in virus-infected plants has been associated with silencing of the virus

genomic RNA by RNA-based antiviral defenses described above. TSV also invades soybean meristems and seed embryos, which results in high levels of seed transmission (Ghanekar and Schwenk, 1974).

The goal of my experiments was to construct a DNA-based VIGS system based on TSV for studying soybean seed development. The TSV-based vector was developed using the TSV RNA2 by inserting plant gene fragments into the 3' terminus of the truncated 2b protein coding region. The length of the gene fragments and inverted repeat sequences required for effective gene silencing were also examined. The small RNA signatures of TSV infected soybean after symptom recovery were also investigated.

MATERIALS AND METHODS

Virus isolate and plant materials

The cDNAs of RNAs 1, 2, 3 and 4 of an Illinois soybean isolate of TSV were synthesized by reverse transcription polymerase chain reaction (RT-PCR) and inserted between the *StuI* and *SaII* restriction enzyme sites downstream of CaMV 35S promoter into the pHST40 vector (Hobbs et al., 2012; Scholthof, 1999) (Fig. 3.1, 3.2). The pHST40 vector has a nopaline synthase (NOS) poly(A) terminator and a *Hepatitis delta virus* antigenomic ribozyme (HDVagrz) to generate a 3' termini similar to wild-type TSV (Scholthof, 1999) (Fig. 3.1). The cDNA clones of the TSV genomic RNA (12.5 µg each) were inoculated biolistically with the Helios Gene Gun system (Bio-Rad, Hercules, CA, USA) with 1.6 µm gold particle at a rate of at least 0.125 mg gold

particle/shot and 1 µg of plasmid DNA/shot (Yamagishi et al., 2006) as described in Chapter 2. The infectivity of the TSV cDNA clones was evaluated on soybean cultivars, ‘Koganejiri’, ‘Itachi’, plant introduction (PI) 88799 and ‘Williams 82’. Inoculated soybean seedlings were grown in 2:1 soil/soilless (Metro-Mix 900, Sun Gro Horticulture Inc., Bellevue, WA) and maintained in the greenhouse at temperatures ranging from 25°C to 27°C and a photoperiod of 12 h.

TSV infections in the inoculated plants were confirmed by tissue-blot immunoassay (TBIA) (Lin et al., 1990) using alkaline phosphatase antibodies specific for TSV (Agdia, Inc., Elkhart, IN) as elaborated in Chapter 2. The cDNA clones of RNA1, RNA2 and RNA3 were sequenced using primers spaced approximately 1 kb apart. The sequencing reactions were performed with Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA) at the University of Illinois, W.M. Keck Center for Comparative and Functional Genomics.

Construction of DNA-based TSV RNA2 VIGS vectors

The TSV-based vector was synthesized from RNA2 by introducing multicloning sites (MCSs) at the end of the truncated coding region of the 2b protein. The 2b protein coding region, produced from an overlapping open reading frame (ORF) with 2a protein, is 614 nt long encoding a protein of 205 amino acid (aa) residues (Fig. 3.3). The deletions in the 3’ terminus of the ORF encoding the 2b and insertion of MCS consisting of the restriction sites *Mlu*I, *Nhe*I and *Xho*I (Fig. 3.4) was carried out using inverse PCR. The primer pairs TSV-R2-2bMR, (5’-TCTCGTCGACACGCGTACCTAGAGCACGAAGCT-3’, *Mlu*I site underlined) and TSV-R2-

2770F (5'-TCTCACGCGTGCTAGCCTCGAGTGATTTAGGTATTCCTCTCTT-3', *Mlu*I, *Nhe*I and *Xho*I sites underlined) were used in the inverse PCR reaction to synthesize pHR2bM, the VIGS vector with a deletion of 144 nt from the ORF encoding the 2b protein in TSV wild-type (Fig. 3.3B). The pHR2bS VIGS vector from RNA2 with a 286 nt deletion in the 2b protein (Fig. 3.3C) and a MCS (*Mlu*I, *Nhe*I and *Xho*I restriction sites) was developed with primer pairs TSV-R2-2bSR, (5'-TCTCGTCGACACGCGTAACCTTGACAGAGAACATC-3', with *Mlu*I site underlined) and TSV-R2-2770F. The inverse PCR with iProof High Fidelity (HF) DNA Polymerase (Bio-Rad, Hercules, CA), a proof reading DNA polymerase, was performed at reaction conditions of 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 66°C for 20 s, and 72°C for 2 min, with a final extension period of 72°C for 10 min. The PCR products were digested with *Mlu*I and ligated with T4 DNA ligase (Promega Corp., Madison, WI) to synthesize the VIGS vectors, pHR2bM and pHR2bS. The truncation of the 2b protein ORF and insertion of the MCS was verified by electrophoresis of the PCR products with primers TSV-R2-2364F (5'-GCAGTTCAATACAGTCAATCCAGTG-3') and TSV-R2-SaIIr (5'-GAGAGTCGACGCATCTTCCATTTGGAGGCATCAGTTA-3') on high resolution gels (Agarose SFR, Amersco, Solono, OH) amplified using 95°C for 2 min; 20 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 2 min; and 72°C for 10 min.

The cDNA clones of RNAs 1, 3 and 4 were mixed with each VIGS vector pHR2bM and pHR2bS and bombarded onto soybean unifoliolate leaves as described for the cDNA clones of the TSV Illinois isolate. Inoculated plants were tested for infection with the mutated TSV by sequencing RT-PCR products and TBIA. Total RNA was extracted from systemically infected foliage tissue using RNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA) and first strand cDNA

was synthesized using Superscript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA) and primer TSV-R2-SaIR. The cDNA was amplified with primers TSV-R2-2364F and TSV-R2-SaIR as described above and sequenced with TSV-R2-2364F.

Soybean cultivars, ‘Williams 82’ (Missouri Foundation Seeds, University of Missouri, Columbia, MO) and ‘Itachi’ (USDA Soybean Germplasm Collection, Urbana, IL), susceptible to TSV were used to evaluate the TSV RNA2-based VIGS vectors, pHR2bM and pHR2bS.

Modifications in TSV RNA1 for construction of TSV VIGS vectors

The RNA1 of TSV is monocistronic and encodes protein 1a that is essential for RNA replication (Scott et al., 1998). To develop a RNA1 based VIGS vector a MCS comprising of *Mlu*I, *Nhe*I and *Xho*I was inserted at nt position 3388 in the 3’ non coding region using inverse PCR and restriction digestion to synthesize the vector pHR1. Primers TSV-R1-3388F (5’-GAGAACGCGTGCTAGCCTCGAGTGTATTTTGCCGGT, *Mlu*I, *Nhe*I and *Xho*I site underlined) and TSV-R1-3355R (5’-GAGAACGCGTGTTCCGAACTTCGCAGAAAATCTGCGGCAA-3’, *Mlu*I site underlined) were used for the inverse PCR performed with iProof HF polymerase at reaction conditions of 98°C for 30 s followed by 20 cycles of 98°C for 10 s, 68°C for 30 s, and 72°C for 5 min, with a final extension period of 72°C for 10 min. The pHR1 clone was developed by *Mlu*I digestion and ligation of the PCR product as in pHR2bM. The insertion of the MCS was verified by electrophoresis on high resolution gels of the PCR product with primers TSV-R1-2841F (5’-ACCACTTACAGATGTCCAAGAGATGCC-3’) and TSV-R1-SaIR (5’-

GAGAGTCGACGCATCTCCTTTAAAGGAGGCATTGTTTATATC-3') amplified using 95°C for 2 min; 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. The pHR1 vector was bombarded into soybean ('Williams 82' and 'Itachi') and the plants were tested for infection with the vector using TBIA and by sequencing RT-PCR products from primers TSV-R1-2841F and TSV-R1-SaIR.

The PDS and MgCh partial gene inserts, PDS-1, PDS-2, PDSstlp-1, PDSstlp-2, MgCh1 and MgCh2 were inserted into pHR1 as described for pHR2bM. The presence and integrity of the inserts in the virus within the inoculated plants were determined by sequencing RT-PCR products amplified with the primers TSV-R1-2841F and TSV-R1-SaIR as described above.

Modifications in TSV RNA3 for construction of TSV VIGS vectors

Several approaches were tested to construct a VIGS vector from TSV RNA3. A vector (pHR3AMV) with the subgenomic promoter that directs the synthesis of RNA4 from *Alfalfa mosaic virus* (AMV), closely related member of the *Bromoviridae*, was inserted in RNA3 upstream of the TSV RNA4 subgenomic promoter and a MCS to insert gene fragments was synthesized downstream of the promoter. The pHR3AMV clone was developed by inserting a MCS comprised of restriction sites for *SpeI*, *BglII*, *MluI*, *NheI*, *XhoI* into RNA3 of TSV at nt position 1147 in the intergenic region between the MP and the CP coding regions (pHR3).

Inverse PCR with primers TSV-R3-1165F (5'-GAGAACGCGTGCTAGCCTCGAGGAGTATTAAGTGGATGAATTCTA-3, *MluI*, *NheI* and *XhoI* sites underlined) and TSV-R3-1151R (5'-

GAGAACGCGTAGATCTACTAGTGTCTCACTCTGGGGGA-3', *Mlu*I, *Bgl*III and *Spe*I sites underlined) was performed at 95°C for 30 s followed by 5 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 3 min 30 s and by another 25 cycles of 95°C for 10 s, and 72°C for 3 min 30 s and a final step of 72°C for 10 min. The PCR product was digested with *Mlu*I, ligated, transformed, screened using primers TSV-R3-842F (5'-GAAGTCGTGCCTCAATAGATGTAG-3') and TSV-R3-1326R (5'-TTCACGGTATTATGGGCGGGACAA-3') with a PCR program of 95°C for 2 min; 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. The oligonucleotides were designed comprising the AMV subgenomic promoter and *Spe*I and *Bgl*III overhang on their 5' and 3' termini, respectively (Invitrogen, Carlsbad, CA). The oligonucleotides comprising the positive and the negative strands (1 mM each) were mixed and annealed by heating at 97°C followed by cooling to 37°C in 10 min. The annealed AMV subgenomic promoter was inserted into the *Spe*I/*Bgl*III sites of pHR3. The clones were screened with primers TSV-R3-842F and TSV-R3-1326R and bombarded into soybean 'Itachi'.

The *Thosea asigna virus* cis-acting hydrolase element (TAV-CHYSEL), was inserted into TSV RNA3 at the 5' and 3' termini of the CP coding region and 5' terminus of the MP coding region along with restriction sites to insert gene fragments to develop VIGS vectors. TAV-CHYSEL can induce breaks in polypeptides by cotranslationally preventing peptide bond formation between glycine and proline amino acids and generating multiple proteins from a single transcript (Trichas et al., 2008). A MCS of *Mlu*I, *Nhe*I, *Xho*I, *Spe*I, and *Bgl*III was inserted at nt position 1253 at the 5' terminus of the CP using primers TSV-R3-1253F (5'-GAGACTCGAGACTAGTAGATCTTCTTCTCGTACTAACAACCG -3', *Xho*I, *Spe*I and *Bgl*III sites underlined) and TVS-R3-1235R (5'-

GAGACTCGAGGCTAGCACGCGTCATGGCGTTGGATGGATG-3', *XhoI*, *NheI* and *MluI* sites underlined). The annealed oligonucleotides with the TAV-CHYSEL sequence were inserted into the *SpeI/BglIII* sites to develop the pHR3TAVCP5' vector using a similar approach as in pHR3AMV. The pHR3TAVCP3' vector with TAV-CHYSEL sequence at the 3' terminus of CP was synthesized by inserting *SpeI*, *BglIII*, *MluI*, *NheI* and *XhoI* at nt position 1925 of TSV RNA3 with primers TSV-R3-1925F (5'-GAGAACGCGTGCTAGCCTCGAGTGACTAGATGGTCACCTCG-3', *MluI*, *NheI* and *XhoI* sites underlined) and TSV-R3-1902R (5'-GAGAACGCGTAGATCTACTAGTATCTTGATTCACCAGAAAATCTT-3', *MluI*, *BglIII* and *SpeI* sites underlined). The TAV-CHYSEL oligonucleotide was inserted into the *SpeI/BglIII* sites. The TAV-CHYSEL was also inserted into the 5' terminus of MP coding region by designing a MCS of *BglIII*, *XhoI*, *NheI* and *MluI* sites at nt position 215 of TSV RNA3 with primers TSV-R3-215F (5'-GGAAAATCCCGGTCCAAGATCTTGCGTTAGTACCAACGATGA-3', *BglIII* site underlined) and TSV-R3-195R (5'-GTGTCTCGAGGCTAGCACGCGTCATCTTCCAATTCAGTGGCG-3', *XhoI*, *NheI* and *MluI* sites underlined). The TAV-CHYSEL sequence was inserted into the *BglIII/XhoI* sites to develop the pHR3TAVMP5'. The TAV-CHYSEL sequence containing RNA3 clones were bombarded into soybean 'Itachi'. The stability of pHR3TAVCP3' was evaluated by electrophoresis of RT-PCR products generated with TSV-R3-842F and TSV-R3-SalIR as described. The stability of pHR3TAVMP5' was analyzed by RT-PCR products generated by TSV-R3-769F (5'-CGTCTCAATCGCCTCGGTTTTATG-3') and TSV-R3-SalIR (5'-GAGAGTCGACGCATCTCCTATAAAGGAGGCATCAGTAGTATATTA-3') using the program 95°C for 2 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 10 min.

Evaluating the effectiveness of TSV RNA2-based VIGS vectors for silencing soybean genes

Soybean genes for phytoene desaturase (PDS) and magnesium chelatase subunit H (MgCh) were selected to evaluate the TSV-based VIGS vectors. Soybean PDS and MgCh gene fragments of sizes varying from 105 bp to 317 bp and inverted repeats of sizes 21 to 50 bp were inserted into the pHR2bM and pHR2bS clones to evaluate the integrity of the insert after inoculation into soybean and their silencing efficiency. PDS, an enzyme in the carotenoid pathway, when silenced produces bleaching of leaves (Bartley et al., 1991). MgCh is an enzyme essential for chlorophyll synthesis and silencing of the gene leads to lower chlorophyll content in leaves producing leaf yellowing (Papenbrock et al., 2000).

The PDS gene fragment of 317 bp was synthesized from nt 35-352 (PDS-1) of the Glyma18g00720.2 soybean mRNA transcript by RT-PCR from total RNA extracted from soybean plants using RNeasy Plant Mini Kit (Qiagen). The cDNA was synthesized with reverse primer PDS-1R-*Xho*I, (5'-GAGACTCGAGGGGAGAGAAATGGTTCCT-3', *Xho*I site underlined) using Superscript II reverse transcriptase. PCR was performed with iProof HF polymerase with forward primer PDS-1F-*Mlu*I (5'-GAGAACGCGTGCCGCTTGTGGCTATATATC-3, *Mlu*I site underlined) and reverse primer PDS-1R-*Xho*I. The thermal cycling program comprised of initial denaturing of 98°C for 30 s followed by 5 cycles of 98°C for 10 s, 53°C for 30 s, and 72°C for 15 s and then by 25 cycles of 98°C for 10 s, 68°C for 30 s, and 72°C for 15 s and a final extension period of 72°C for 10 min. The PCR products and the vectors pHR2bM and pHR2bS were digested with *Mlu*I and *Xho*I.

The *MluI-XhoI* digested PCR product was ligated into the vectors and verified by PCR using primer pairs TSV-R2-2364F and TSV-R2-SalIR.

Two other inserts (~155 bp) from Glyma18g00720.2 , PDS-2 and PDS-3, were generated using primer pairs PDS-2F-Mlu (5'-GAGAACGCGTATGCCAAATAAGCCTGGAGA-3', *MluI* site underlined), / PDS-2R-Xho (5'-GAGACTCGAGAAGCATAGCTGGGAGAAG-3', *XhoI* site underlined); and PDS-3F-Mlu (5'-GAGAACGCGTGCTGATGCTGGGCATAAACCT-3', *MluI* site underlined), / PDS-3R-Xho (5'-GAGACTCGAGGCCAAGTTCTCCAAAAAGG-3', *XhoI* site underlined), respectively. The PCR conditions used for PDS-2 insert were 95°C for 2 min followed by 5 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 15 s and by 25 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 15 s and a final extension period of 72°C for 10 min. The PCR thermal cycle for PDS-3 was similar to PDS-2 with the annealing temperatures of 53°C for 5 cycles and 66°C for 25 cycles. The inserts were generated from 383-541 nt and 587-739 nt regions of Glyma18g00720.2 with *MluI* and *XhoI* sites on the 5' and 3' termini, respectively. The *MluI-XhoI* digested fragment was inserted into the *MluI* and *XhoI* sites in the MCS of pHR2bM and pHR2bS and verified using primers TSV-R2-2364F and TSV-R2-SalIR.

Similarly, two MgCh gene fragments (nt 576-750 and nt 3410-3514) flanked by *MluI* and *XhoI* sites on their 5' and 3' ends, respectively, were synthesized from soybean MgCh subunit H transcript Glyma03g29330.1 and inserted into MCS of the TSV-based VIGS vector. The first 175 bp MgCh partial gene insert (MgCh-1) was constructed with primers MgCh-1F-Mlu, (5'-GAGAACGCGTGACTTGGAGGATGCCAACAT-3', *MluI* site underlined) and MgCh-1R-Xho (5'-GAGACTCGAGGCTGTGACATGCTGAAGGAA-3', *XhoI* site underlined). A second 105

nt MgCh partial gene insert (MgCh-2) was generated using MgCh-2F-Mlu (5'-GAGAACGCGTTGGAAGGCCTAGGATTGATG-3', *MluI* site underlined) and MgCh-2R-Xho (5'-GAGACTCGAGTCAGCAACCATCTTCACTGC-3', *XhoI* site underlined). The PCR conditions were 95°C for 2 min followed by 5 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 15 s and by another 25 cycles of 95°C for 30 s, 66°C for 30 s, and 72°C for 15 s and a final step of 72°C for 10 min.

Two inverted repeats, PDSstlp-1 and PDSstlp-2 (Fig. 3.5), of 21- and 23-nt corresponding to nt 1208-1229 and nt 1009-1032 of the predicted Glyma18g00720.2 transcript, were synthesized as complementary oligonucleotides with *MluI* and *XhoI* overhang on their 5' and 3' termini, respectively (Invitrogen, Carlsbad, CA). The oligonucleotides comprising the positive and the negative strands were annealed as in pHR3AMV and inserted into pHR2bM. A stem loop of 50-nt long was also synthesized that represented nt 35 - 85 transcript Glyma18g00720.2 (Invitrogen). The vectors pHR2bM and pHR2bS with the partial gene inserts were inoculated by particle bombardment as described above.

Determining the integrity of the partial plant gene inserts in the TSV RNA2-based VIGS vector

The presence and the integrity of the inserts in the virus within the inoculated plants were determined by sequencing RT-PCR products amplified with the primers TSV-R2-2364F and TSV-R2-SaIIR as described above and by northern blots with psoralen-biotin-labeled probes. The RT-PCR and northern blots were performed on total RNA extracted (RNeasy Plant Mini

Kit, Qiagen) from the infected plants a month after inoculation. The sequencing of the RT-PCR product was conducted following the same protocol as for TSV-based VIGS vectors, pHR2bM and pHR2bS as mentioned above.

Psoralin-biotin-labeled probes for the northern blots were designed using BrightStar® Psoralen-Biotin Kit (Ambion, Austin, TX) from PCR products, PDS-2, PDS-3 and MgCh-1, generated using primer pairs PDS-2F-Mlu/PDS-2R-Xho, PDS-3F-Mlu/ PDS-3R-Xho and MgCh-1F-Mlu/MgCh-1R-Xho, respectively, as described before. Probes were labeled as directed in the protocol for BrightStar® Psoralen-Biotin Kit.

For northern blot analysis 1 µg of total RNA (RNeasy Plant Mini Kit, Qiagen) in RNA loading buffer (Ambion) was denatured at 100°C and electrophoretically separated on 1% agarose gels in diethyl pyrocarbonate (DEPC)-treated tris-acetate-EDTA buffer at 105V for 45 min. Gels were trimmed to remove any unused lanes and transferred onto nylon membranes (Amersham Hybond-N+, GE Healthcare Ltd, Little Chalfont, UK) overnight with a transfer buffer (5×SSC, 10 mM NaOH). Nylon membranes were neutralized in 0.2 M sodium phosphate buffer for 4 min, dried for 50 min at 80°C and UV-crosslinked on both sides.

Membranes were pre-hybridized for 1 hr at 42°C in ULTRArray™ hybridization buffer (Ambion) followed by hybridization overnight at 42°C in the same buffer with added denatured probes (100°C for 5 min) for PDS-2, PDS-3 and MgCh-1. Membranes were washed twice at low stringency in 2×SSC, 0.5% sodium dodecyl sulfate (SDS) at 42°C for 30 min each, followed by three washings in blocking buffer (BB, 0.2% Tropix I block (Applied Biosystems Inc, Foster

City, CA), 1×tris-buffered saline (TBS) and 0.5% SDS) for 5 min each at room temperature. Membranes were placed in 1:5000 dilution of Avidx-APTM (Tropix, Bedford, MD), alkaline phosphatase-conjugated streptavidin, in blocking buffer for 30 min. Membranes were then washed thrice in 1×TBS, 0.5% SDS for 5 min each and given two final rinses of 5 min each in assay buffer (0.1 M diethanolamine, 1.0 mM MgCl₂) to prepare the membranes for binding to CDP-StarTM (New England BioLab, Ipswich, MA), a chemiluminescent alkaline phosphatase substrate. Membranes were soaked in CDP-Star for 5 min and the excess substrate solution was drained off and membranes were wrapped in plastic wrap and exposed to X-ray films for 5 hr.

Estimation of gene silencing of inserted gene by TSV RNA2-based VIGS vector

The gene silencing was estimated using quantitative (q)RT-PCR (Power SYBR Green RNA-to-CT 1-Step Kit, Applied Biosystems) by quantifying the changes in levels of mRNA in soybean plants inoculated with TSV-based VIGS vector with the gene fragment when compared to plants inoculated with the VIGS vector without any partial plant gene insert (Livak and Schmittgen, 2001). The expression of the gene of interest was normalized to a housekeeping gene, actin (cytoskeleton protein; Glyma19g00850.1).

The primers for real-time qRT-PCR estimation of PDS and MgCh mRNA levels were designed from regions in the transcript sequence that was not directly targeted by the gene fragments inserted in the VIGS vector. The primer pairs PDS-RT-F (5'-CTTTGGCATTCTTTGTCTGTGG-3') and PDS-RT-R (5'-TTATTGCACACAGTTGACGACG-3') that amplified the 1795 to 1914 nt region in the

soybean Glyma18g00720.2 transcript were used to detect PDS gene transcription levels. The qRT-PCR primers for the soybean MgCh genes were designed from the central region of the transcript sequences (MgCh-RT-F: 5'-CTATGCTGCAAACAACCCTTCT-3' and MgCh-RT-R: 5'-AYAGYCCWGCATTTTCMGC-3'). The primer pairs Act1289F (5'-CCTGATGGGCAGGTTATCACT-3') and Act1349R (5'-GGTACAAGACCTCCGGACAC-3') measured the soybean actin gene (Glyma19g00850.1) transcript levels used as a reference gene to normalize for the amount of RNA template. The thermal cycling conditions for real-time RT-PCR were 48°C for 30 min for reverse transcription and 95°C for 10 min for activation of the polymerase followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation curve was generated at the end of the cycle to detect non-specific amplification (95°C for 15 s, 60°C for 15 s and 95°C for 15 s).

Seed transmission

Seed transmission rates of unmodified TSV infectious cDNA clones were evaluated in soybean cultivars 'Koganejiro', 'Itachi' and 'PI 88799'. The seed transmission of pHR2bM and pHR2bS, with the MCS, were verified by TBIA and sequencing of RT-PCR products using primers TSV-R2-2364F and TSV-R2-SalIR as described earlier. Seed transmission rates of TSV-pHR2bM and TSV-pHR2bS VIGS vectors with PDS and MgCh inserts, were evaluated in soybean 'Williams 82'. The seed transmission rates were determined by grow-out tests by planting mature seeds from TSV infected plants in 72-well polystyrene trays containing soilless mix (Sunshine Mix LC1; Sun Gro Horticulture Inc., Bellevue, WA). The presence of TSV infection in the seedlings in grow-out tests was determined by TBIA at the unifoliate stage and the presence of the partial

plant gene insert in the truncated RNA2 vector was confirmed by electrophoresis on high resolution gels of RT-PCR product synthesized by primers TSV-R2-2364F and TSV-R2-SalIR.

Small RNA signature sequencing of TSV-infected plants

The small RNA profiles of soybean plants infected with TSV exhibiting symptom recovery were analyzed by deep sequencing. Unifoliate seedlings of soybean ‘Itachi’ and ‘Williams 82’, were mechanically inoculated with plant extracts (prepared in 10 mM sodium phosphate buffer, pH 7) from TSV-infected plants. Total RNA was extracted from the first leaf showing symptom recovery in TSV-infected plants and mock inoculated plants from leaves at the same growth stage of soybean ‘Itachi’ using *mirVana*TM miRNA Isolation Kit (Invitrogen). The small RNAs of 20 to 25 nt were purified using polyacrylamide denaturing gel. The small RNA was sequenced using a Roche Genome Analyzer II at Cofactor Genomics, St Louis, MO. The small RNA sequencing of soybean ‘Williams 82’ infected with TSV and mock inoculated plants were carried out on RNA extracted using the *mirVana*TM Kit following the enrichment procedure for small RNA. Small RNAs were sequenced using Illumina Genome HiSeq 2000 at the University of Illinois, W. M. Keck Center for Comparative and Functional Genomics. Adapter sequences were trimmed from the sequences generated and were aligned to TSV genome using bowtie (Langmead et al., 2009) and analyzed using PERL and Microsoft Excel 2007.

RESULTS

Development of infectious cDNA clone of an Illinois soybean isolate of TSV

An infectious cDNA clone of Illinois soybean isolate of TSV was successfully constructed by inserting RNAs 1, 2, 3 and 4 into the pHST40 cloning vector. The clone had a CaMV 35S promoter obviating the need for *in vitro* RNA transcript production for inoculation and making it conducive to direct inoculation. The clones were highly infectious with all biolistically inoculated plants showing symptoms of TSV infection. The soybean cultivars, ‘Koganejiro’, ‘Itachi’ and ‘PI 88799’, exhibited severe symptoms such as, mosaic, leaf curling and necrosis, followed by symptom recovery (Fig. 3.6). Foliar symptoms on soybean cultivar ‘Williams 82’ were mild on the initial leaves with mosaic and leaf curling, and subsequent leaves were symptom free. The infectious clone was sequenced and the sequences of RNAs 1, 2, and 3 were submitted to GenBank (FJ403375, FJ403376, and FJ403377, respectively) (Hobbs et al., 2012). The TSV-based VIGS vectors were developed by modifying the cDNA clones.

Infectivity of TSV-based VIGS vectors

The MCS of *Mlu*I, *Nhe*I and *Xho*I was successfully inserted into the truncated RNA2 of TSV to develop TSV-based vectors, pHR2bM and pHR2bS. The TSV-based VIGS vector pHR2bM was highly infectious and produced symptoms and symptom recovery indistinguishable from the wild-type RNA2. The vector pHR2bS also had high infectivity with all inoculated plants becoming infected, but the infected plants remained symptomatic. The integrity of the MCS

present in the TSV-based vector inoculated plants was preserved during seed transmission, which was confirmed by sequencing virus recovered after seed transmission from soybean cultivars 'Itachi' and 'Williams 82'. The TSV RNA1-based vector pHR1 was infective and the MCS was retained in the infected plants but all the partial plant gene inserts were deleted from the virus genome during replication in soybean. The TSV RNA3-based vectors lost the inserted AMV promoter and TAV-CHYSEL sequences from the virus genome during infection and spread in soybean.

Integrity of partial plant gene inserts in the TSV RNA2-based VIGS vectors

The stabilities of inserts in soybean plants inoculated with TSV-based VIGS vectors pHR2bM and pHR2bS, carrying PDS and MgCh gene fragments of varying sizes, were analyzed. The cDNA clones were confirmed by sequencing before inoculation into soybean. The 317-bp PDS insert into pHR2bM and pHR2bS failed to produce any infection in soybean 'Williams 82' whereas in soybean cultivar 'Itachi' the insert was progressively deleted from the TSV genome during virus multiplication and spread within the plant. The pHR2bM with the PDS-2 (152-nt) (pHR2bM-PDS2), PDS-3 inserts (158-nt) (pHR2bM-PDS3), MgCh-1 (175-nt) (pHR2bM-MgCh1) and MgCh-2 (105-nt) were highly infectious (Table 3.1) and the integrity of the inserts was maintained in 'Williams 82' (Fig. 3.7) and infected plants exhibited TSV-wild type symptoms. The soybean cultivar 'Williams 82' was also successfully infected with TSV VIGS vector pHR2bS with PDS-2, PDS-3, MgCh-1 inserts (Table 3.1; Fig. 3.8). One of nine plants infected with pHR2bS-PDS3 exhibited partial yellowing of the leaves indicative of gene silencing of PDS genes about 30 days post inoculation. The inverted repeats of PDS gene,

PDSstlp-1 and PDSstlp-2, inserted into pHR2bM were lost from the vector in soybean cultivar 'Itachi' and the larger inverted repeat was not stable in the pHR2bM or pHR2bS cDNA clones and was not analyzed.

Silencing of plant genes with TSV RNA2 based VIGS vectors

The relative transcript levels ($\Delta\Delta Ct$) from PDS and MgCh genes were evaluated by comparing mean cycle thresholds for plants inoculated with pHR2bM-PDS2, pHR2bM-PDS3 and pHR2bM-MgCh1 to plant inoculated with the empty vector, pHR2bM. Similarly, plants inoculated with pHR2bS-PDS2, pHR2bS-PDS3 and pHR2bS-MgCh1 were compared to plants inoculated with the empty vector, pHR2bS, in 'Williams 82'. Down-regulation of PDS transcript levels was observed only in one of nine plants infected with pHR2bS-PDS3 (Table 3.2) with an expression of half the level as plants inoculated with pHR2bS. The results indicated that the silencing was sequence specific, but was not efficient or consistent.

Seed transmission

Transmission rates of TSV cDNA infectious clones through seeds varied from 0 to 95% depending on the soybean cultivar. The soybean cultivar 'PI 88799' had the highest seed transmission rate of 95% and the cultivars 'Itachi' and 'Koganejro' transmitted TSV through seed at rates of 39% and 0%, respectively. In the soybean cultivar 'Williams 82' the seed transmission rates of pHR2bM-MgCh1 was 8% and the inserted gene fragment of maximum 175

nt size was retained in the pHR2bM vector after seed transmission. The seed transmission of pHR2bS-PDS3 and pHR2S-MgCh1 were 3% in 'Williams 82'.

Small RNA signature sequencing of TSV infected plants

The sequencing of small RNAs from TSV-infected and mock-inoculated soybean 'Itachi' and 'Williams 82' generated 2.4 million to 16 million sequences. The comparison of small RNA sequence abundance between the different treatments was done after normalizing and expressed as hits per million reads (hpmr). The proportion of total small RNAs of size 21-nt in TSV infected 'Itachi' signature was 21% higher than mock inoculated 'Itachi' and the siRNAs of size 24-nt were 15% lower than signatures from mock inoculated plants (Table 3.3) Whereas in the small RNA signatures of 'Williams 82', the 21-nt small RNAs from TSV-infected plants were 2% more abundant than from mock inoculated plants and the 24-nt small RNAs were 6% lower in TSV-infected than in mock-inoculated plants (Table 3.3). TSV-infected 'Itachi' had a higher proportion of small RNA sequences (29%) that mapped to the TSV genome than TSV-infected 'Williams 82'. As most small RNA sequences were between the sizes of 21 to 24, all the remaining analysis were conducted with this group of small RNAs.

The virus-derived (v) siRNA signature sequences from TSV-infected soybean aligned in higher proportions to the sense strand than the antisense strand with 72% and 61% in 'Itachi' and 'Williams 82' signatures, respectively aligned to the sense strand. The GC content in the vsiRNA varied from 44% in 'Itachi' to 46% in 'Williams 82' which is similar to the TSV GC content of 44% indicating no bias of GC content in vsiRNA production. The first nucleotide at the 5' end of

the vsiRNA showed a bias toward U, with vsiRNA aligning to TSV having U at the 5' end constituting 36% in 'Itachi' and 30% in 'Williams 82' (Fig. 3.10). The nucleotide at the 3' end also showed a bias toward U, with 30% of vsiRNA in 'Itachi' and 32% of vsiRNA in 'Williams 82' having U at the 3' end (Fig. 3.11). The 21-nt vsiRNAs were the most predominant and the 24-nt siRNA population was the least abundant (Fig. 3.9). The signature sequencing revealed that vsiRNAs were produced nonuniformly from TSV RNAs (Figs. 3.12, 3.13, and 3.14). While no vsiRNAs were generated from some regions of TSV RNAs, others generated many copies of sRNA to a maximum of 14,600 hpmr. For comparison, the soybean miRNAs, *gma-miRNA159a* and *gma-miRNA166* that were overrepresented in 'Itachi' and 'Williams 82' had 18,351 hpmr and 240,908 hpmr, respectively. Most small RNA hot spots to the TSV genome of more than 200 hpmr were located in RNA3, with 77% and 53% of such hot spots (> 200 hpmr) in 'Itachi' and 'Williams 82', respectively, aligning to TSV RNA3.

In signature sequences from TSV infected 'Itachi', there were 140 hot spots (>200 hpmr) that mapped to the TSV genomic RNA. There were 17 (12%) hot spots (>200 hpmr) in TSV RNA1 from 'Itachi', with 2 mapping to the 5' noncoding region and 15 mapping to the 1a coding region (Fig. 3.12A). In Itachi, hot spots in TSV RNA2 (Fig. 3.13A) mapped to the 2a (14 hot spots) and 2b (1 hot spot) coding regions and in TSV RNA3 (Fig. 3.14A) mapped to 5' noncoding region (31 hot spots), MP coding region (58 hot spots) and CP encoding region (16 hot spots). In 'Williams 82', 34 hot spots mapped to TSV genomic RNA of which 11 mapped to RNA1 (2 in 5' noncoding region and 9 in 1a coding region), 5 mapped to RNA2 (1 in 5' noncoding region and 4 in the 2a coding region) and 18 mapped to RNA3 (4 in 5' the noncoding region, 12 in MP coding region and 2 in the CP coding region) (Figs. 3.12B, 3.13B, and 3.14B).

DISCUSSION

Highly infectious cDNA clones of TSV RNAs were constructed in the pHST40 vector, downstream of a CaMV 35S promoter and upstream of the HDVagrz and NOS transcription terminator. The CaMV 35S promoter facilitates the production of a DNA-based VIGS vector that can be directly inoculated into soybean, circumventing the need for linearization of the cDNA clone and RNA transcript production. The HDV ribozyme enabled the formation of authentic stem-and-loop structures at the 3' termini of the RNAs that are present in wild-type TSV genomic RNAs. The stem-and-loop structures at the 3' end with interspersed AUGC motifs binds with CP in ilarviruses, that is necessary for translation initiation and therefore infection (Krab et al., 2005; Reusken and Bol, 1996). The pHST40 vector has also been utilized to develop cDNA clones of viruses lacking poly(A) at the 3' terminus such as *Tomato bushy stunt virus* and AMV (Balasubramaniam et al., 2006; Scholthof, 1999). Seed transmission of TSV has been recorded to be cultivar dependent ranging from 2.6 to 36% (Ghanekar and Schwenk, 1974). The infectious TSV cDNA clone was seed transmitted at rates as high as 95% in soybean 'PI 88799' indicating invasion of the reproductive meristem.

The TSV-based VIGS vectors, pHR2bM and pHR2bS, with the MCS of *Mlu*I, *Nhe*I and *Xho*I restriction sites for inserting heterologous gene fragments were highly infectious. The integrity of the MCS was maintained through seed transmission making the vectors accessible for functional genomics of genes expressed in soybean seed. Fragments of PDS and MgCh up to 175-nt were inserted into the TSV-based VIGS vector and were preserved in the infected plants and through seed transmission.

Silencing was established in only a few plants, although the inserts were maintained and spread throughout the plants. The lack of silencing of the targeted endogenous genes could have resulted from suppression of RNA silencing by the TSV silencing suppressor in the infected plants (Voinnet et al., 1999). However, TSV has not been reported to express a strong silencing suppressor and TSV-infected plants exhibit symptom recovery, which is often associated with silencing of virus by plant antiviral defense mechanism (Baulcombe, 2005). Based on similarity to CMV, also from the same family (*Bromoviridae*), the 2b protein of TSV is the protein most likely to suppress RNA silencing (Brigneti et al., 1998; Xin et al., 1998). This study indicated that a larger deletion from the 3' terminus of the 2b gene resulted in decreased silencing and loss of symptom recovery, which also implicates 2b protein in RNA silencing suppression. Similar to the 2bS clone, Xin and Ding (2003) identified a naturally occurring mutant of TSV that was defective in the initiation of recovery. Xin and Ding (2003) observed that symptom recovery in TSV was associated with lower accumulation of TSV genomic RNA and coat protein. However, the single-nucleotide change responsible for the lack of recovery was located in the intergenic region of RNA3 at the transcriptional start site of RNA4, which encodes the CP. Hence, this and the study of Xin and Ding (2003) suggest that recovery from infection in TSV is actively mediated by sequences in two different TSV RNAs and not simply the result of the lack of a strong silencing suppressor. Deletions of sequences encoding the carboxyl terminal 16 amino acids of the CMV 2b protein produced mutant viruses that exhibited persistent severe symptoms in infected tobacco plants and the truncated 2b failed to localize to nuclei as does the wild-type protein (Lewsey et al., 2009). Since CMV 2b localizes to the nucleus and is required for cell-to-cell movement and meristem invasion in addition to suppressing RNA silencing, if TSV 2b has

similar functions, it is possible that loss of one of these other functions alter host responses without affecting RNA silencing.

The vsiRNAs of TSV mapped in higher proportion to the sense strand similar to *Cymbidium ringspot virus*, *Tobacco mosaic virus* (TMV) and *Tobacco rattle virus* (TRV) (Donaire et al., 2008; Qi et al., 2009; Szittyta et al., 2010). The higher production of vsiRNAs from the sense strands can be due to high abundance of sense strands or to preferential loading of sense strand vsiRNAs into RISC complexes (Donaire et al., 2008). Most vsiRNA hot spots, with alignment of more than 200 siRNAs hpmr, mapped to TSV RNA3 and to the MP-encoding region within TSV RNA3. The production of vsiRNA at hotspots was not associated with secondary structures in TSV RNA3 predicted by Mfold (Zucker, 2003). The lack of association of vsiRNAs with obvious secondary structures in virus RNAs has also been recorded in TMV and TRV (Donaire et al., 2008; Qi et al., 2009). The vsiRNAs were predominantly of 21-nt in size indicating that vsiRNAs were mainly processed by DCL-4 and AGO1 or AGO2 proteins (Blevins et al., 2006; Deleris et al., 2006; Garcia-Ruiz et al., 2010; Mi et al., 2008; Morel et al., 2002). The U residue at the 5' termini of TSV siRNAs also suggested the role of AGO1 protein in the production of vsiRNA in TSV (Mi et al., 2008) as has been reported from other viruses (Donaire et al., 2008; Qi et al., 2009; Szittyta et al., 2010). The proportion of siRNA from the TSV genome in 'Itachi' and 'Williams 82' accounted for 29% and 10%, respectively and the siRNA from BPMV, another virus that is silenced in soybean (Lim et al., 2011), were 11% and 14% in 'Itachi' and 'Williams 82'. The proportion of hot spots with alignment of more than 200 siRNA hpmr were also very low in the 2b coding region which might lead to lower siRNA production of any insert in that region hence making it ineffective in silencing. The accumulation of TSV vsiRNAs was

much lower than that of some endogenous miRNAs, which may account for the low levels of gene silencing observed. However, through careful selection and design of plant gene-specific inserts it may be possible to enhance the production of siRNAs from the inserted sequence and hence the effectiveness of the TSV VIGS vectors. Alternatively, the TSV RNA2-based vectors are highly infectious with systemic movement within soybean making them suitable for use as expression vectors to study gene functions.

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TABLES

Table 3.1. The proportion of plants inoculated with *Tobacco streak virus* (TSV) virus-induced gene silencing (VIGS) vectors, pHR2bM and pHR2bS, with partial plant gene inserts for phytoene desaturase (PDS) and magnesium chelatase (MgCh) that had the insert stably multiplied and systemically spread in soybean cultivar ‘Williams 82’.

TSV VIGS vectors	Proportion of plants infected
pHR2bM	86% ^a (6/7) ^b
pHR2bM-PDS2	100% (4/4)
pHR2bM-PDS3	100% (4/4)
pHR2bM-MgCh1	45% (4/7, 2/6)
pHR2bS	100% (8/8)
pHR2bS-PDS2	100% (8/8)
pHR2bS-PDS3	100% (8/8)
pHR2bS-MgCh1	100% (8/8)

^a percentage of plant infected

^b (number of plants infected / number of plants inoculated)

Table 3.2. The relative accumulation of phytoene desaturase (PDS) or magnesium chelatase (MgCh) mRNAs in soybean plants infected with in the *Tobacco streak virus* (TSV) virus-induced gene silencing (VIGS) vector, pHR2bS, inserted with PDS or MgCh gene fragments in comparison with pHR2bS (empty vector).

TSV VIGS vectors	$\Delta\Delta Ct$	$2^{-\Delta\Delta Ct}$
pHR2bS- PDS2	-0.2 ± 0.3^a	1.1 (1.4 - 0.9) ^b
pHR2bS-PDS3	1.0 ± 0.3	0.5 (0.6 - 0.4)
pHR2bS-MgCh1	0.1 ± 0.2	1.0 (1.1 - 0.8)

^a $\Delta\Delta Ct$ (relative expression of PDS gene in pHR2bS- PDS2) = (Mean of pHR2bS-PDS2 Ct_{PDS} - Mean of pHR2bS-PDS2 Ct_{Actin}) - (Mean of pHR2bS Ct_{PDS} - Mean of pHR2bS Ct_{Actin}).

^b $2^{-\Delta\Delta Ct}$ is folds or the relative amount of PDS transcripts in pHR2bS-PDS2 to pHR2bS.

Table 3.3. The size distribution of small RNA sequences from *Tobacco streak virus* (TSV) infected and mock inoculated soybean, ‘Itachi’ and ‘Williams 82’.

siRNA size	TSV infected Itachi	Mock Inoculated Itachi	TSV infected Williams 82	Mock inoculated Williams 82
<20	7%	10%	6%	5%
21	59%	38%	40%	38%
22	14%	10%	21%	23%
23	3%	6%	4%	5%
24	14%	29%	15%	20%
>24	4%	8%	14%	8%

FIGURES

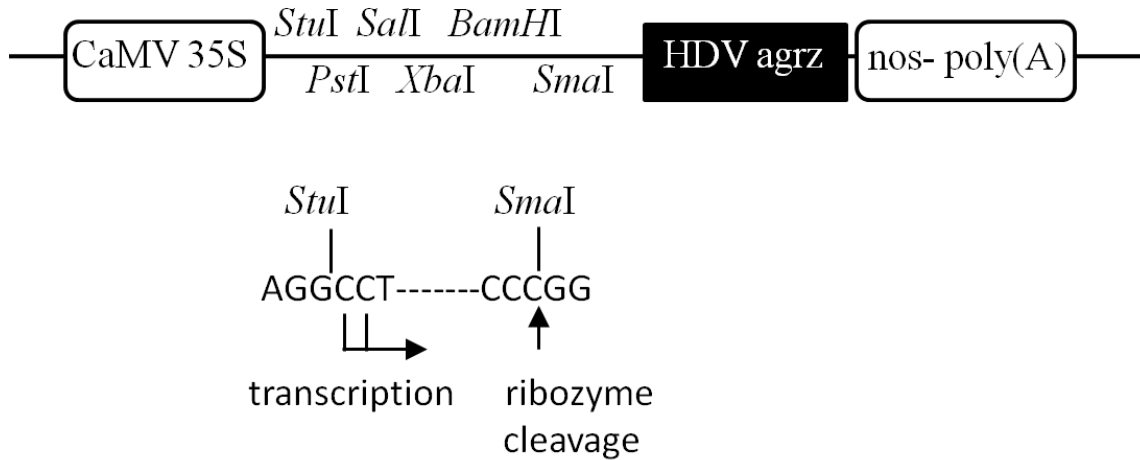


Figure 3.1. The pHST40 vector (Scholthof, 1999) used in the construction of infectious cDNA clones of *Tobacco streak virus* RNAs, which were inserted downstream of the *Cauliflower mosaic virus* (CaMV) 35S and upstream of the *Hepatitis delta virus* antigenomic ribozyme (HDVagrz) and nopaline synthase poly(A) terminator (nos-polyA) between the *StuI* and *SalI* sites.

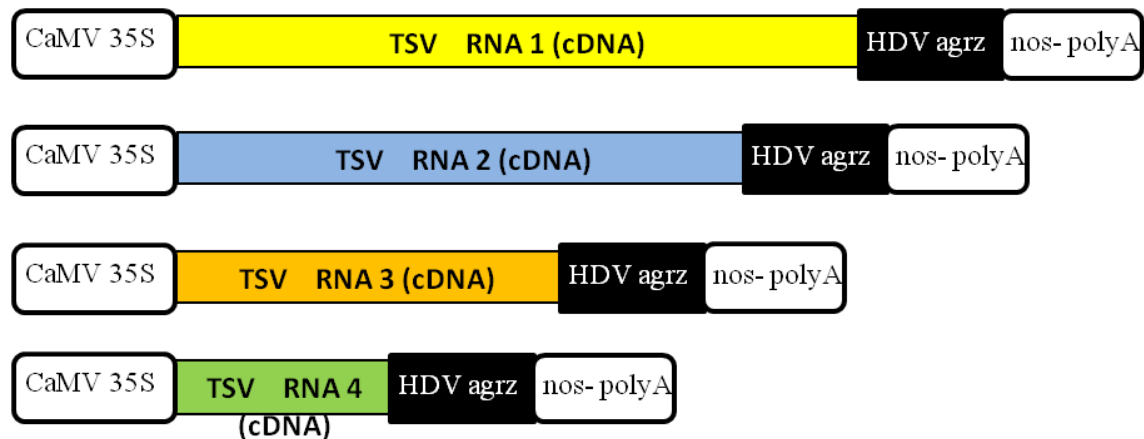


Figure 3.2. The cDNA clones of *Tobacco streak virus* (TSV) RNAs 1, 2, 3 and 4 in pHST40 with *Cauliflower mosaic virus* (CaMV) 35S promoter at the 5' terminus and *Hepatitis delta virus* antigenomic ribozyme (HDVagrz) and nopaline synthase poly(A) terminator (nos-polyA) at the 3' terminus.

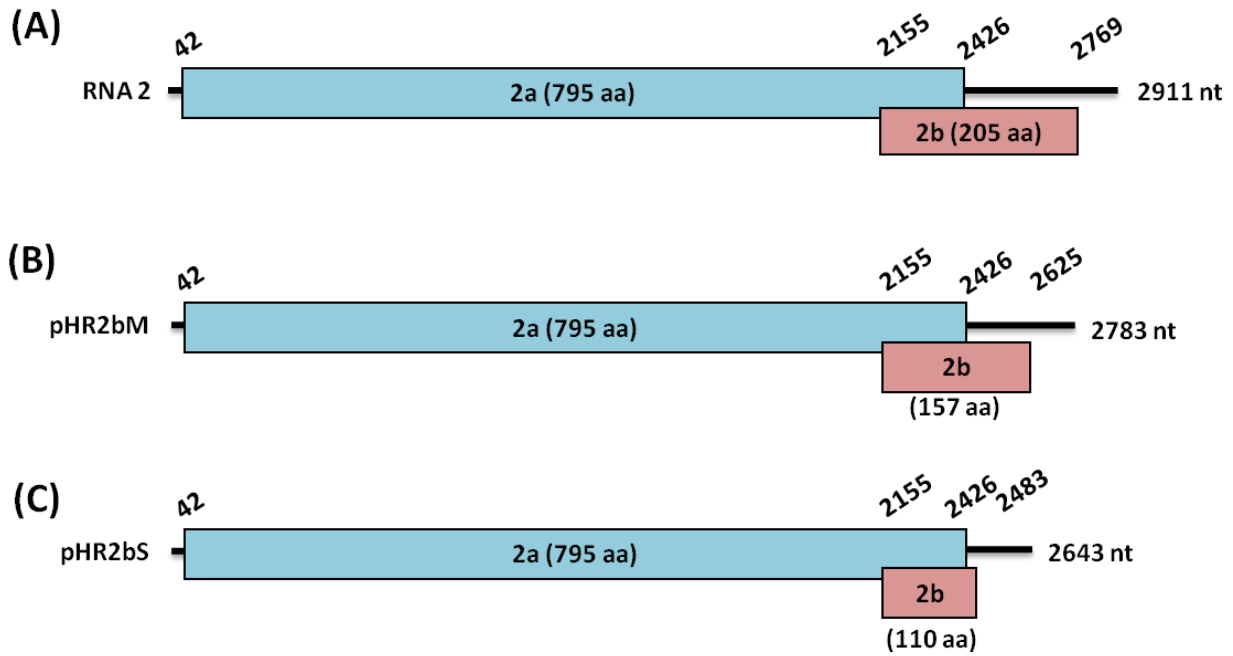


Figure 3.3. Structure of *Tobacco streak virus* (TSV) RNA2 and vectors for virus-induced gene silencing (VIGS) with truncated 2b protein coding regions (A) The RNA2 of TSV has 2911 nucleotides (nt) with overlapping open reading frames (ORFs) of 2a with 796 amino acid (aa) residue and 2b protein (205 aa). (B) The pHR2bM of 2783-nt with truncated 2b of 157 aa (C) The pHR2bS with truncated 2b protein with a deletion of 286-nt.

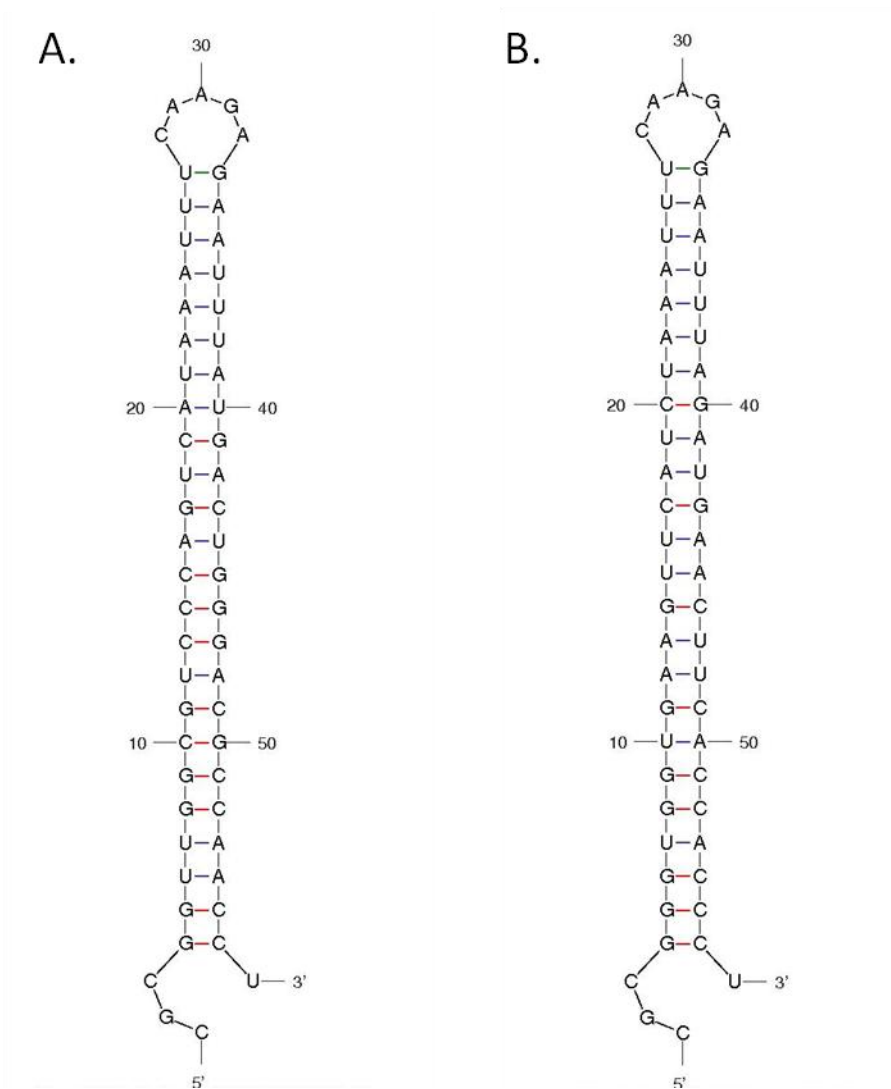


Figure 3.5. The stem-loop inserts of phytoene desaturase (PDS) gene (transcript Glyma18g00720.2). A. PDSstlp-1 corresponding to nt 1208-1229 and B. PDSstlp-2 corresponding to nt 1009-1032 of Glyma18g00720.2.



Figure 3.6. Soybean ('Itachi') plant biolistically inoculated with *Tobacco streak virus* showing mosaic and necrosis followed by symptom recovery of subsequent emerging leaves.

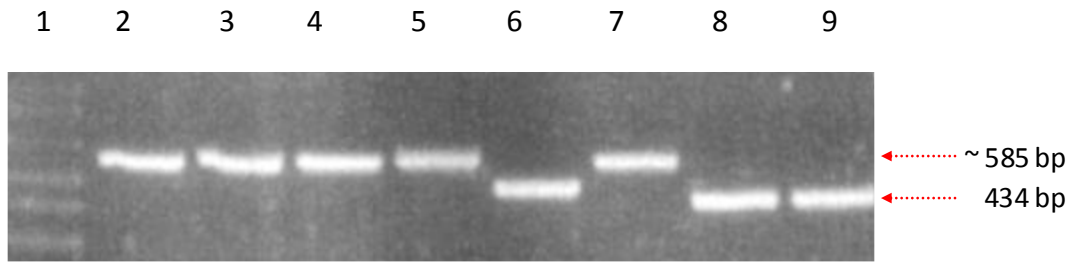


Figure 3.7. Separation by high resolution agarose gel electrophoresis of reverse transcriptase polymerase chain reaction (RT-PCR) products amplified from primer pairs TSV-R2-2364F and TSV-R2-SalIR from soybean inoculated with the pHR2bM *Tobacco streak virus* (TSV) virus-induced gene silencing (VIGS) vector with soybean phytoene desaturase (PDS) partial gene inserts and pHR2bM empty vector. Lanes 2-3: RT-PCR product from soybean plants inoculated with pHR2bM-PDS2 (~ 585 bp); lane 4: PCR product from cDNA clone pHR2bM-PDS2 (~ 585 bp) as positive control; lanes 5-6: RT-PCR product from soybean plants inoculated with pHR2bM-PDS3; lane 7: PCR product from cDNA clone pHR2bM-PDS3 as positive control (~ 585 bp); lane 8: RT-PCR product from soybean plants inoculated with pHR2bM (empty vector); lane 9: PCR product from cDNA clone pHR2bM (empty vector) (434bp).

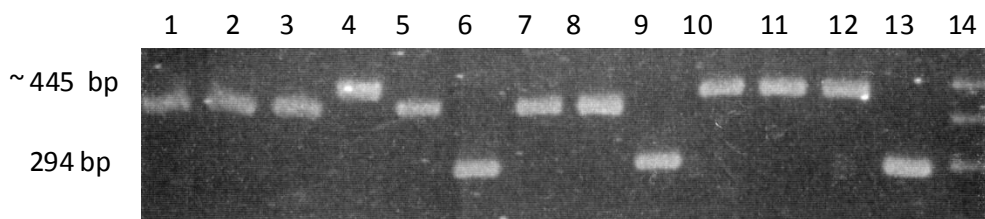
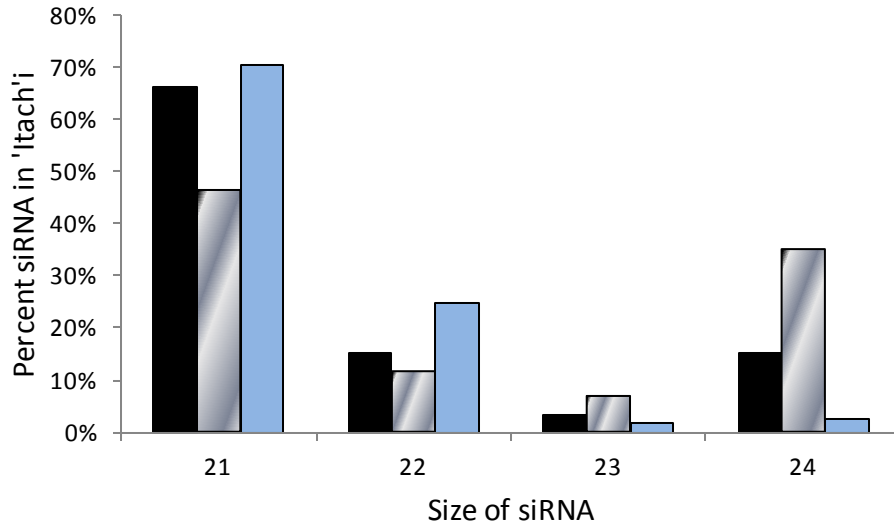


Figure 3.8. The high resolution gel electrophoresis analysis of reverse transcriptase polymerase chain reaction (RT-PCR) products to verify multiplication and spread of *Tobacco streak virus* virus-induced gene silencing vectors pHR2bS with soybean phytoene desaturase (PDS) and magnesium chelatase subunit H (MgCh) partial gene inserts (PDS-2, PDS-3 and MgCh-1) in soybean using primer pairs TSV-R2-2364F and TSV-R2-SallR. Lane 1-4: RT-PCR product from soybean plants inoculated with pHR2bS-PDS2 (~ 445 bp); lanes 5-8: RT-PCR product from soybean plants inoculated with pHR2bS-PDS3 (~ 445 bp); lanes 9-12: RT-PCR product from soybean plants inoculated with pHR2bS-MgCh1 (~ 470 bp); lane 13: PCR product from the cDNA clone pHR2bS (empty vector)

A.



B.

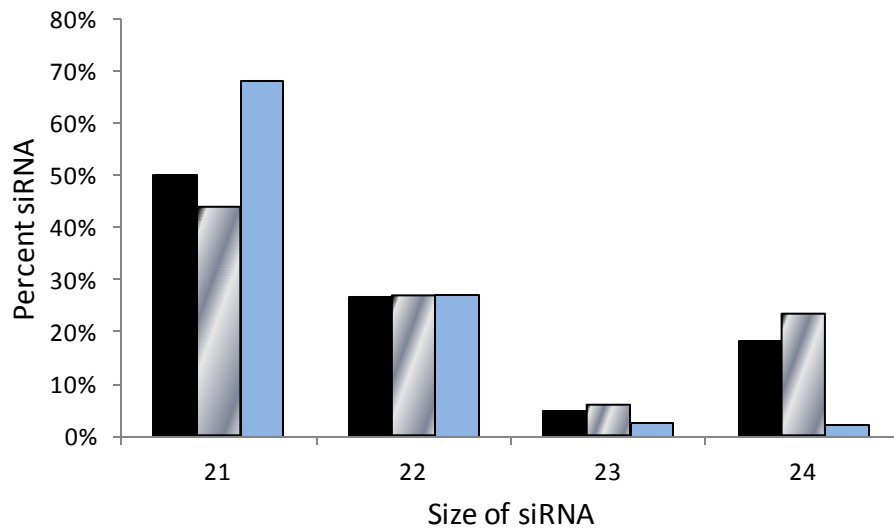


Figure 3.9. Proportions of small interfering RNAs of different sizes in *Tobacco streak virus* (TSV)-infected (black bars), mock inoculated (gray bars) soybean, and in TSV-infected soybean that mapped to the TSV genome (blue bars) A. in soybean cultivar ‘Itachi’ B. in soybean cultivar ‘Williams 82’.

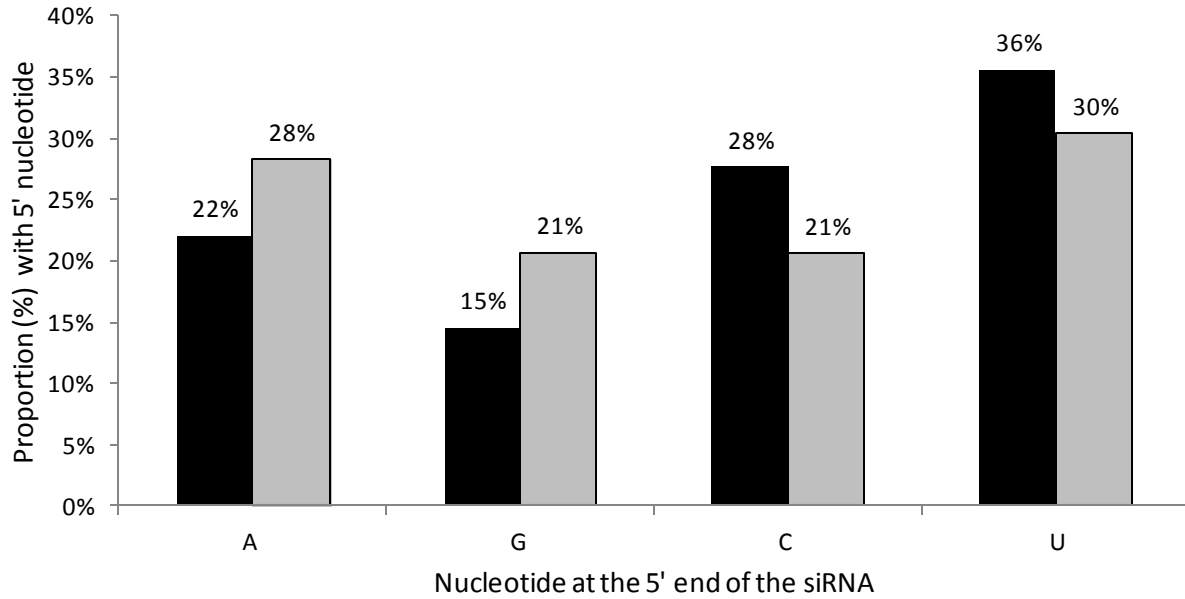


Figure 3.10. Proportions of small interfering RNAs that mapped to the *Tobacco streak virus* genomic RNAs with 5' termini nucleotide (A, G, C, U as indicated) in 'Itachi' (black bars) and 'Williams 82' (gray bars)

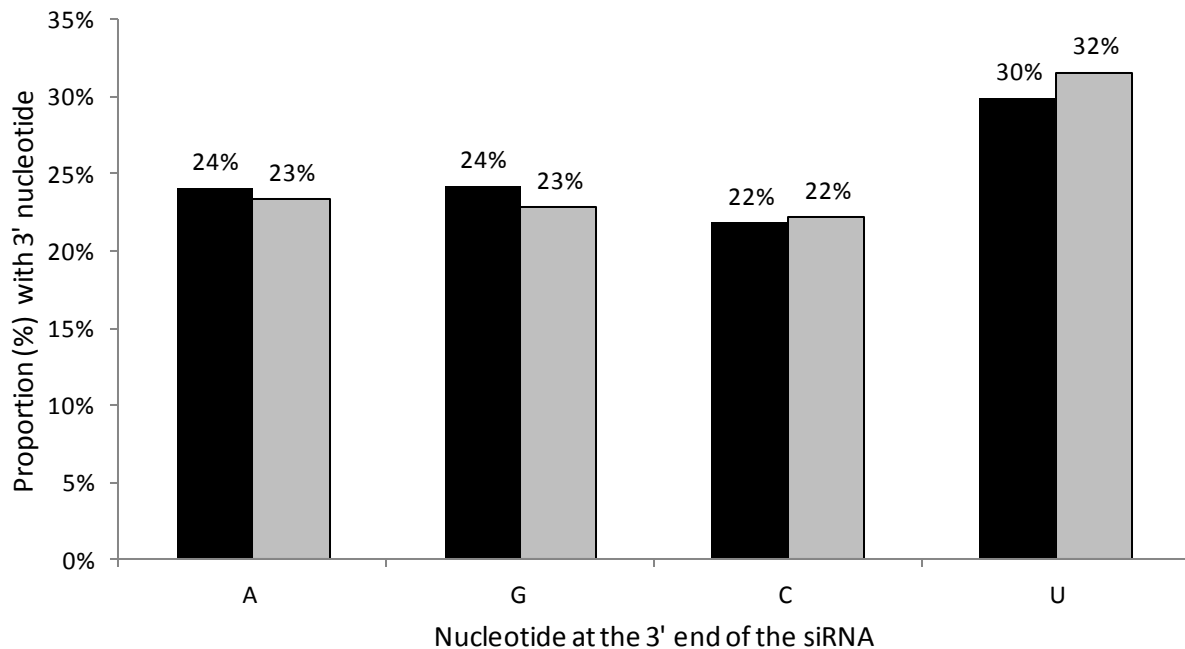
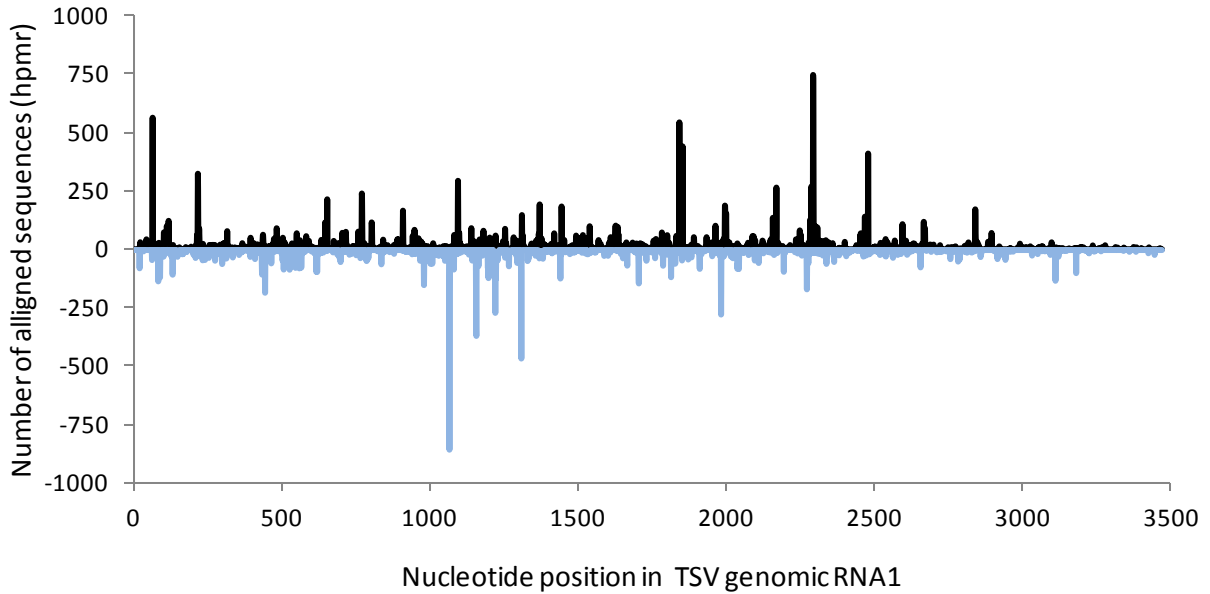


Figure 3.11. Proportions of small interfering RNAs that mapped to the *Tobacco streak virus* genomic RNAs with 3' termini nucleotide (A, G, C, U as indicated) in 'Itachi' (black bars) and 'Williams 82' (gray bars)

A.



B.

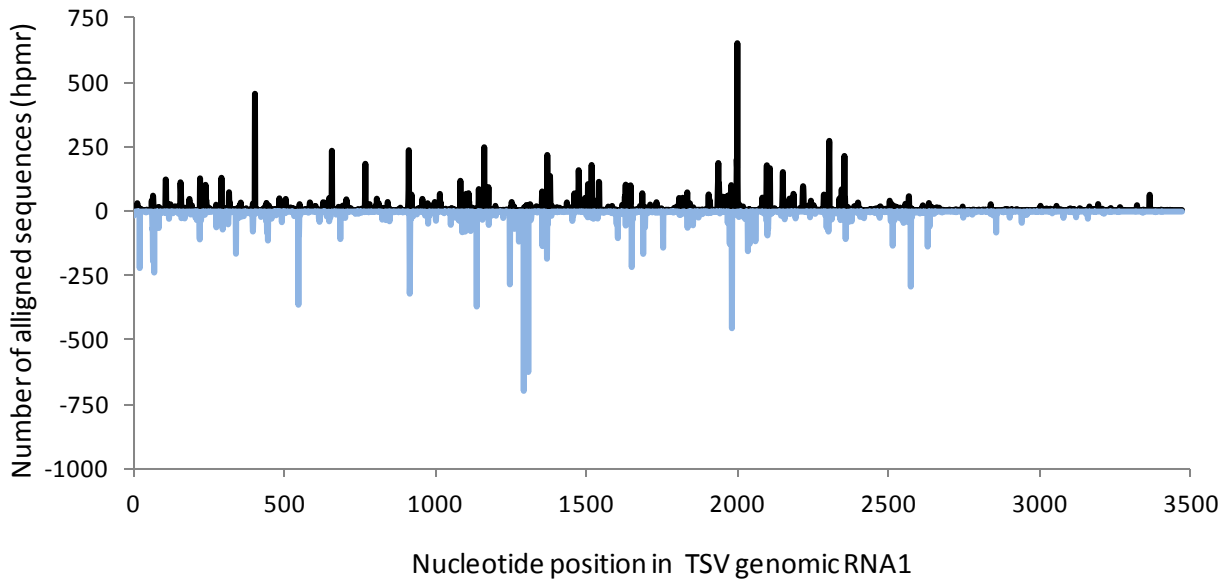
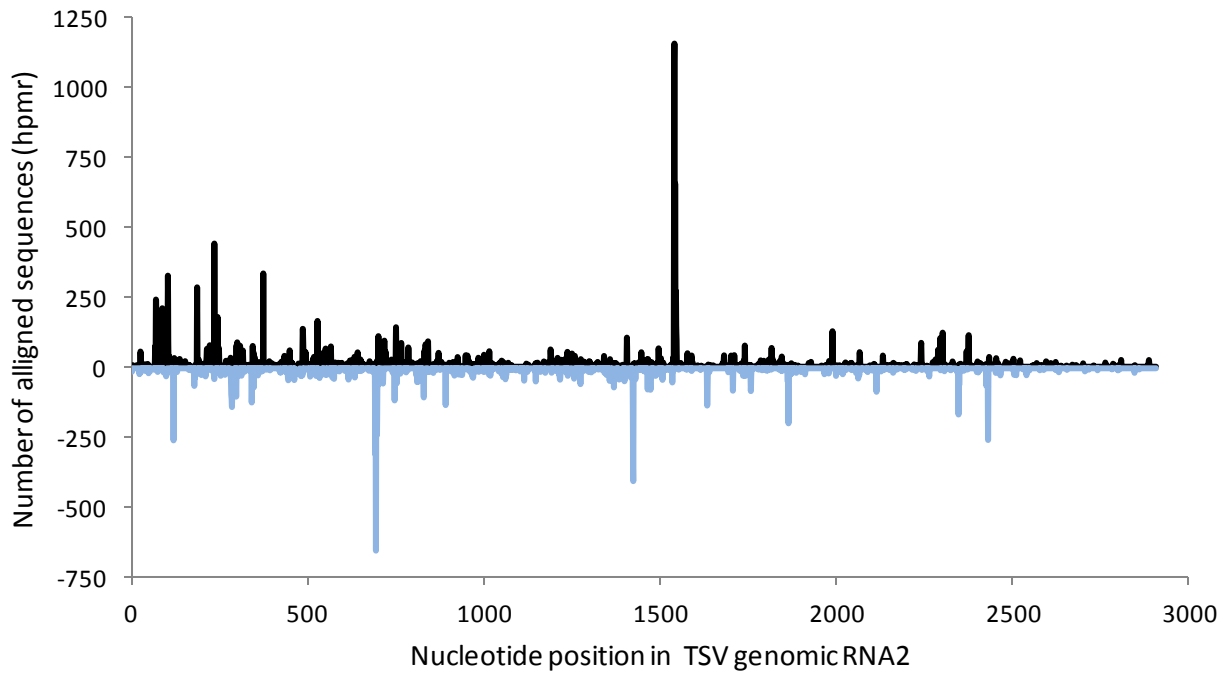


Figure 3.12. Normalized (hpmr, hits per million reads) abundance of small interfering RNA sequences in soybean infected with *Tobacco streak virus* (TSV) that mapped to the positive (above X-axis) and negative strands (below X-axis) of the genomic TSV RNA1. A. in soybean cultivar 'Itachi' and B. in soybean cultivar 'Williams 82'

A.



B.

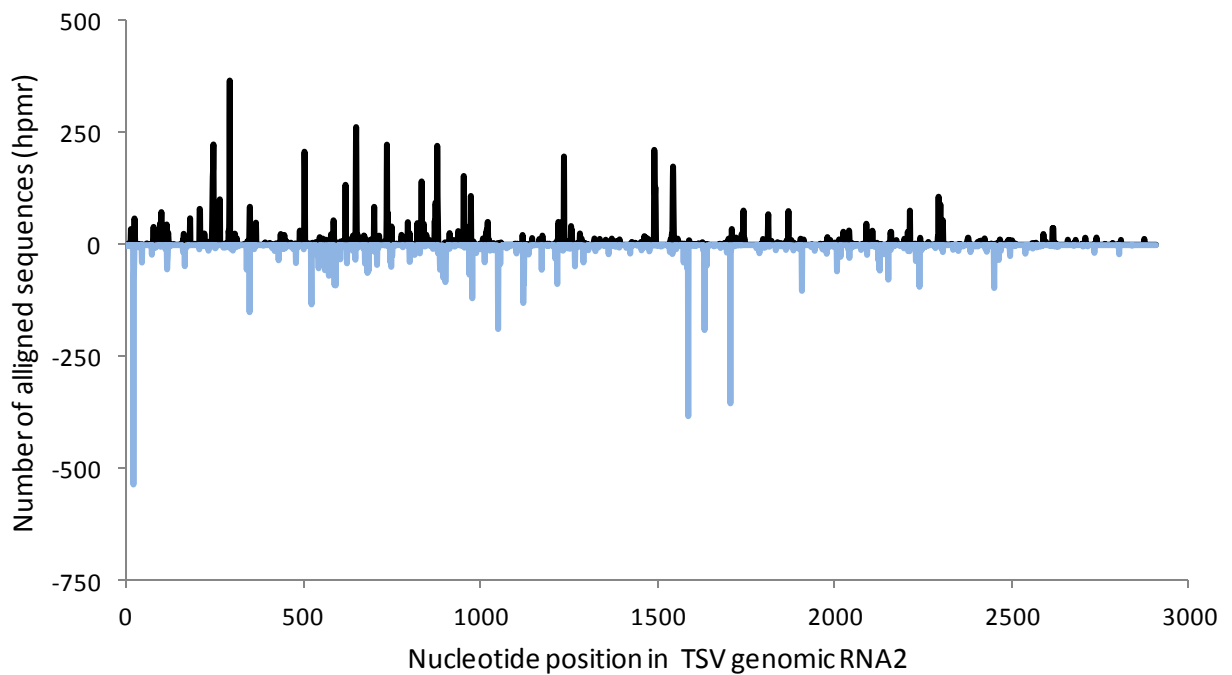
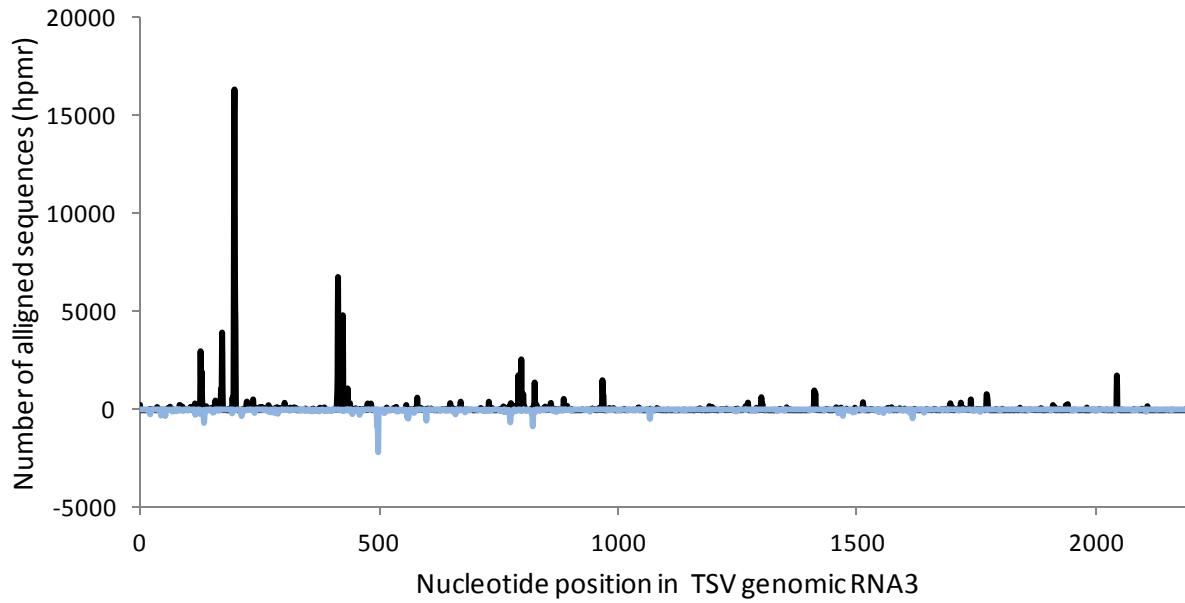


Figure 3.13. The number of small interfering RNA sequences, after normalization to 1 million (hpmr, hits per million reads), in soybean infected with *Tobacco streak virus* (TSV) that mapped to the positive (above X-axis) and negative strands (below X-axis) of the genomic TSV RNA2. A. in soybean cultivar ‘Itachi’ and B. in soybean cultivar ‘Williams 82’.

A.



B.

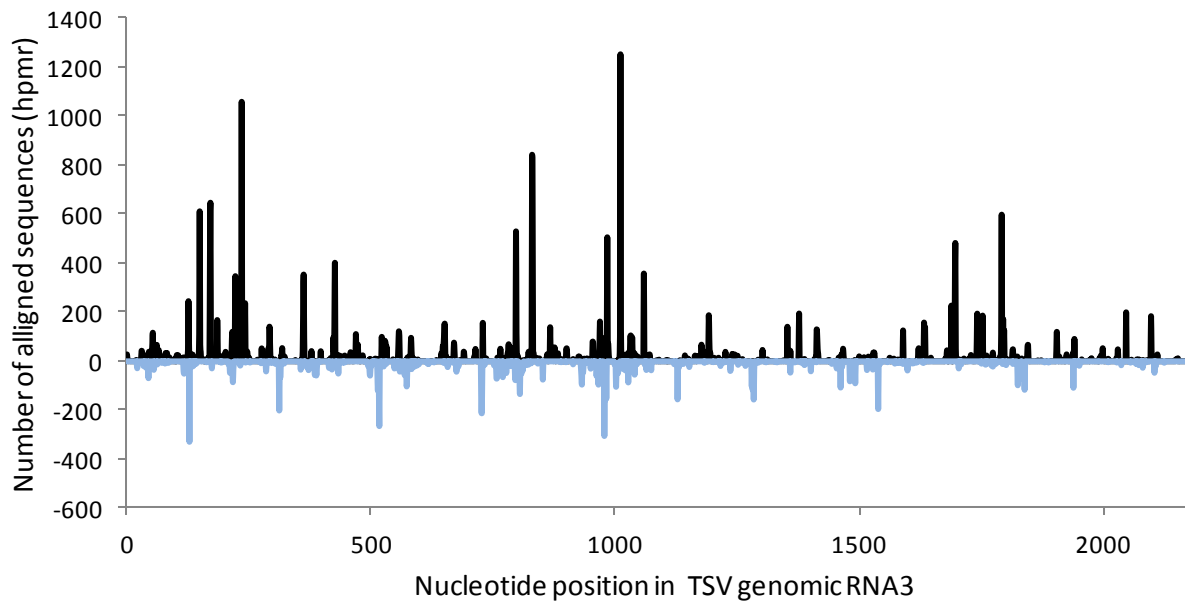


Figure 3.14. Normalized abundance (hpmr, hits per million reads) of small interfering RNA sequences in soybean infected with *Tobacco streak virus* (TSV) that mapped to the positive (above X-axis) and negative strands (below X-axis) of the genomic TSV RNA3. A. in soybean cultivar 'Itachi' and B. in soybean cultivar 'Williams 82'.

CHAPTER 4

MICRORNA AND SOYBEAN GENES INVOLVED IN PLANT-VIRUS INTERACTIONS DURING SYMPTOM RECOVERY IN VIRUS INFECTED SOYBEAN

ABSTRACT

Infection of soybean with either *Tobacco streak virus* (TSV) or *Bean pod mottle virus* (BPMV) results in severe initial symptoms that are followed by symptom recovery. The symptom recovery is associated with posttranscriptional silencing of virus genomes. To counteract host RNA-based antiviral defenses, many plant viruses express suppressors of RNA silencing that also alter the accumulation and activities of host microRNAs (miRNAs). In this study, the accumulation of plant mRNAs and miRNAs in soybean cultivars ‘Itachi’, ‘Mumford’ and ‘Williams 82’ infected with TSV or BPMV and showing symptom recovery were compared to those of mock-inoculated plants by deep sequencing of small RNAs and RNA-Seq analysis. The soybean miRNAs *gma-miRNA159a*, which targets transcripts of glutathione S-transferase (GST) and MYB family transcription factors, and *gma-miR166*, which targets HD-Zip transcription factors, were down-regulated in virus-infected soybean tissues exhibiting symptom recovery. The GST and MYB and HD-Zip transcription factor proteins are all involved in programmed cell death that is often associated with plant disease resistance. The mRNA-Seq analysis revealed that the expression of pathogenesis-related proteins, salicylic acid-mediated defense response proteins and RNA-virus infection-associated proteins were up-regulated in virus-infected soybean. Also transcripts for GST and lactoylglutathione lyase protein, involved in

detoxification, and chalcone synthase were down-regulated in virus-infected soybean tissues with symptom recovery. Changes in the accumulations of other defense-related and photosynthetic genes were soybean cultivar dependent, as indicated by the number of sequences that mapped to the transcripts.

INTRODUCTION

MicroRNAs (miRNAs) are small endogenous plant RNAs predominantly of 21 nucleotides (nt) in length that originate from single stranded (ss)RNAs processed from local hairpin structures that act in *trans* to regulate mRNA abundance and/or translation (Llave et al., 2002; Rhoades et al., 2002). MiRNAs play crucial roles in plant development, cell-fate differentiation (Rhoades et al., 2002) and in response to biotic and abiotic stress (Jones-Rhoades and Bartel, 2004; Lia et al., 2012; Mallory and Vaucheret, 2006).

In *Arabidopsis thaliana*, miRNAs are processed from the hairpins in primary transcripts, pri-miRNA, by DCL1 (a RNase III-like enzyme), HYPONASTIC LEAVES1 (HYL1, double-stranded (ds) RNA binding protein) and SERRATE (SE, zinc-finger protein) in the nucleus (Dong et al., 2008; Kurihara and Watanabe, 2004; Papp et al., 2003; Park et al., 2002; Reinhart et al., 2002; Vazquez et al., 2004a). The miRNA/miRNA* duplexes are methylated by HUA ENHANCER 1 (HEN1, methyltransferase protein) and one strand, miRNA, is selectively loaded onto ARGONAUTE 1 (AGO1, RNA endonuclease) that directs the cleavage of complementary mRNA strands or translation repression (Brodersen et al., 2008; Vaucheret et al., 2004; Yu et al., 2005). HASTY, (HST, homologous to Exportin-5) is involved in transport of the miRNA from

the nucleus to the cytoplasm (Bollman et al., 2003; Lund et al., 2004). MiRNAs can also induce the production of trans-acting small interfering (ta-si)RNA, that are generated from double-stranded RNA synthesized by the action of RNA-dependent RNA polymerase (RDR6) and suppressor of gene silencing (SGS3) (Vazquez et al., 2004b). Ta-siRNAs are produced from the mRNAs targeted by the miRNA. The ta-siRNA like siRNA can guide the cleavage or translational repression of complementary mRNA (Allen et al., 2005).

Virus suppressors of gene silencing interfere with miRNA production or function either by inhibiting their activities or by altering their accumulation (Kasschau et al., 2003). Virus silencing suppressors P1/helper component protease (P1/HC-Pro; TuMV, *Turnip mosaic virus*), p19 (TBSV, *Tomato bushy stunt virus*) and p12 (*Beet yellow virus*) can interfere with mRNA cleavage by miRNA by inhibiting the degradation of miRNA* strand (the strand of the miRNA duplex unincorporated into AGO1) (Chapman et al., 2004). Expression of the TBSV p19 silencing suppressor in transgenic plants and infection of *Nicotiana benthamiana* plants by *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX) or *Tobacco etch virus* induce the production of miR168 that down-regulates AGO1 a critical plant protein in RNA silencing (Varallyay et al., 2010). MiR168 is also up-regulated in *Turnip crinkle virus*- and *Ribgrass mosaic virus*-infected *Arabidopsis thaliana*, *Sunn-hemp mosaic virus*-infected *Medicago truncatula* and TMV- and PVX-infected *Solanum lycopersicum* (Varallyay et al., 2010).

Tobacco streak virus (TSV), from the genus *Ilarvirus* and family *Bromoviridae*, causes severe symptom initially on soybean followed by symptom recovery associated with post transcriptional silencing of the virus genomic RNA (Baulcombe, 2005; Fulton, 1978; Fusaro et al., 2006). A

single nucleotide change in the intergenic region of TSV RNA3 resulted in the loss of symptom recovery (Xin and Ding, 2003). *Bean pod mottle virus* (BPMV) a member of the genus *Comovirus* and family *Comoviridae* also causes severe symptoms initially followed by symptom recovery in soybean that is accompanied with a concomitant decrease in accumulation in BPMV genomic RNA in the infected tissue by RNA silencing (Lim et al., 2011).

Here, I present the results from using deep sequencing of small RNAs to assess changes in miRNA levels in leaf tissues of soybean cultivars ‘Itachi’, ‘Mumford’, and ‘Williams 82’ infected with TSV or BPMV that exhibited symptom recovery compared to mock-inoculated plants of the same cultivars. I have also compared transcript abundance in deep sequencing data generated by mRNA-Seq from the same soybean cultivars infected with either TSV or BPMV or mock-inoculated.

MATERIALS AND METHODS

Viruses and plant materials

Unifoliate seedlings of soybean cultivars ‘Itachi’, ‘Mumford’ and ‘Williams 82’ were mechanically inoculated with plant extracts (prepared in 10 mM sodium phosphate buffer, pH 7.0) from systemic leaves of either TSV- or BPMV-infected plants. Virus infections in soybean plants were verified by tissue blot immunoassay (TBIA) with alkaline phosphatase antibodies for TSV and BPMV (Agdia, Inc., Elkhart, IN) as described in Chapter 2 (Lin et al., 1990). RNA was extracted from the first leaf showing symptom recovery in TSV- and BPMV-infected plants and

from leaves at the same growth stage in mock-inoculated plants. Total RNA was extracted from TSV/BPMV-infected or mock-inoculated ‘Itachi’ using *mirVana*TM miRNA Isolation Kit (Invitrogen, Carlsbad, CA) and small (20-25 nt) RNAs were gel purified and sequenced as described in Chapter 3. In soybean cultivars, ‘Mumford’ and ‘Williams 82’, the small RNAs were extracted using the *mirVana*TM Kit followed by enrichment for small RNAs. The partial sequencing of mRNA with mRNA-Seq was performed from total RNA extracted from ‘Mumford’ and ‘Williams 82’ using *mirVana*TM Kit. The small RNA and the mRNA samples were sequenced using Illumina Genome HiSeq 2000 at the University of Illinois, W.M. Keck Center for Comparative and Functional Genomics.

Sequence analysis

Small RNA sequences trimmed of the adapter sequence were aligned to soybean miRNA stem-loop sequences from plant miRNA database (Zhang et al., 2010) using bowtie (Langmead et al., 2009). The miRNA stem-loop sequences were used for alignment to identify changes in abundance of the miRNA and miRNA* strands along with miRNA isoforms between small RNA signatures. Bowtie output was further analyzed using custom PERL scripts and Microsoft Excel 2007. For comparisons between mapped sequences to miRNA stem loop sequences in the signatures, the numbers of mapped sequences were normalized to 1 million (hits per million reads, hpmr) to avoid bias due to the differences in numbers of signature sequences obtained from each sample. The mRNA data were aligned to publically available soybean transcript sequences (Schmutz et al., 2010) using bowtie and analyzed using custom PERL scripts and Microsoft Excel 2007. For comparisons of the numbers of sequences mapped to mRNAs, the

numbers of mapped sequences were normalized to fragments per kilobase of exon per million fragments mapped (fpkm) to avoid bias due to transcript sizes differences and the numbers of signature sequences obtained from each sample.

Northern blot for siRNA

Biotin-labeled oligonucleotide probes (5'-CTCAAAGGGGTGACCTGAGAACACAA-3') for miRNA398 were synthesized (Invitrogen). A probe for soybean U6 small nuclear (sn)RNA (99 bp), which is implicated in splicing of mRNA precursors, was also developed to be used as a loading control. The U6 snRNA probe of 59 bp was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR) using primers U6snRNA-F1 (5'-ACAGAGAAGATTAGCATGGCCCCTG-3') and U6snRNA-R1 (5'-GACCATTCTCGATTTGTGCGTGTC-3') from total RNA extracted from soybean. The thermal cycle for the PCR was 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 15 s and a final 72°C for 10 min. The RT-PCR product was labeled with psoralin-biotin (BrightStar® Psoralen-Biotin Kit).

The change in miRNA accumulation in TSV infected 'Itachi' and 'Mumford' was measured by fragmenting extracted RNA (denatured at 100°C for 5 min) on 15% polyacrylamide/8M urea gel (0.05% ammonium persulfate, 0.09% N'-tetramethylethylenediamine (TEMED), 1×tris-borate electrophoresis buffer at 22 amp for ~30 min). RNAs were transferred to Hybond-N+ membrane (Amersham) using a Bio-Rad Trans-Blot apparatus (Bio-Rad) at 5 V for 60 min. The membranes were dried for 40 min at 80°C and UV-crosslinked on both sides. Blots were probed and washed

at low stringency and treated with CDP-StarTM (chemiluminescent alkaline phosphatase substrate) and exposed to X-ray films for 18-24 h as described in Chapter 3.

RESULTS

Symptom recovery and small RNA deep sequencing

Seedlings of soybean cultivars ‘Itachi’, ‘Mumford’ and ‘Williams 82’ were all successfully infected by TSV or BPMV. The soybeans infected with the virus exhibited leaf curling and mosaic in the first two leaves followed by symptom recovery. TSV-infected ‘Itachi’ exhibited very severe symptoms including bud blight that was followed by symptom recovery (Fig. 3.6).

The deep sequencing of small RNA generated signatures of 2.5 to 26 million sequences with an average of 8 million signatures from the 9 treatments: TSV-infected, BPMV-infected and mock-inoculated soybean cultivars ‘Itachi’, ‘Mumford’ and ‘Williams 82’. The majority (~85%) of the small RNAs were between the sizes of 21 to 24 nt (Fig. 4.1), so all the remaining analysis was conducted with the small RNA of sizes 21 to 24 nt from mock-inoculated plants and soybean tissues infected with TSV or BPMV showing symptom recovery. The small RNAs of 21-nt were the most abundant in all the signatures except for ‘Williams 82’ infected with BPMV where 22-nt small RNAs were the most abundant. Signatures from TSV-infected ‘Itachi’ plants had a large increase in 21-nt small RNAs (Table 4.1). In the signatures from the virus-infected soybeans, the proportions of 22-nt small RNAs were larger than from mock-inoculated soybeans and 24-nt small RNAs were less abundant in virus-infected soybean plants, to a larger extent in TSV-

infected soybean than BPMV-infected plants, than in mock-inoculated plants. The proportion of small RNAs that mapped to the soybean genome was lower in virus-infected plants than mock-inoculated plants as was expected (Table 4.1).

MiRNA population in the small RNA signature

The numbers of sequences that aligned to miRNAs in the small RNA signatures were compared to identify changes that were induced by virus infection followed by symptom recovery. The proportions of sequences that mapped to the miRNA stem-loop structure were lower in small RNA signatures from virus-infected than mock-inoculated plants (Table 4.1).

Changes in miRNA levels in BPMV-infected plants

The number of sequences that mapped to the miRNA stem-loop sequences revealed that most miRNAs were present in approximately equal numbers in all the signatures. Three miRNAs were down-regulated and one miRNA and a miRNA* were up-regulated in BPMV-infected soybean, as indicated by the number of small RNA sequences that mapped to miRNA sequences (Table 4.2). The gma-miR159d that targets glutathione S-transferase (GST) mRNA (Zhang et al., 2008) and MYB family transcription factors (Song et al., 2011; Wong et al., 2011) was up-regulated in BPMV-infected soybeans. However, gma-miR159a and gma-miR159a-isoform, which normally are more abundant than gma-miR159d and can also target GST mRNA, were down-regulated in ‘Mumford’ and ‘Williams 82’ and marginally down-regulated in ‘Itachi’. GST is important for detoxification and redox buffering (Edwards et al., 2000; Foyer and Noctor, 2011).

The gma-miR166 that targets HD-Zip transcription factors that are associated with abiotic stress responses in *Arabidopsis* (Ariel et al., 2007), and gma-miR167 that targets auxin response transcription factors (Song et al., 2011; Zhang et al., 2008) were down-regulated in BPMV-infected soybean compared to mock-inoculated plants. The levels of gma-miR168 and gma-miR168-isoform that target mRNA encoding AGO proteins, which are crucial for virus silencing (Song et al., 2011), were higher in BPMV-infected ‘Mumford’ and ‘Williams 82’ than in the mock inoculated. Interestingly, the gma-miR168* accumulated at a higher level in all BPMV-infected soybean cultivars tested. The levels of gma-miR1507b were also lower in BPMV-infected ‘Mumford’ and ‘Williams 82’ than in mock-inoculated plants of the same cultivars. The levels of gma-miR396, which targets mRNAs of cysteine proteases involved in metabolism (Subramanian et al., 2008; Zhang et al., 2008), was lower in BPMV-infected ‘Itachi’ and ‘Williams 82’ compared to the mock-inoculated plants, but was at higher levels in BPMV-infected than mock-inoculated ‘Mumford’.

Changes in miRNA levels in TSV- infected plants

The gma-miR159a and gma-miR159a-isoforms, which target mRNAs encoding GST and MYB family transcription factors (Song et al., 2011; Wong et al., 2011; Zhang et al., 2008), were also present in lower levels in signatures from TSV-infected than mock-inoculated soybean (Table 4.3). MiRNA, miR398, which targets mRNA superoxide dismutases (SOD), was up-regulated in TSV-infected ‘Itachi’, which was confirmed semi-quantitatively using northern blots (Fig. 4.2), and isoforms of gma-miR398 and gma-miR398* were up-regulated in TSV-infected plants of the

three soybean cultivars (Table 4.3). SOD is an enzyme that is involved in scavenging reactive oxygen species (ROS) and is down-regulated during abiotic stress and hypersensitive resistance response in *Arabidopsis* (Jagadeeswaran et al., 2009). MiR164, which targets mRNA of NAC family transcription factors that are induced during abiotic and biotic stresses (Guo et al., 2005; Nakashima et al., 2007; Song et al., 2011), and miR396 that targets cysteine protease transcripts were down-regulated in TSV-infected ‘Itachi’ and ‘Williams 82’. However in TSV-infected ‘Mumford’, gma-miR396 was up-regulated in leaves exhibiting symptom recovery.

Changes in miRNA levels in soybean cultivars infected with virus

In signatures from virus-infected ‘Itachi’ that exhibited symptom recovery, gma-miR159a, gma-miR164, gma-miR166, gma-miR169, gma-miR396, and isoforms of gma-miR1508 and gma-miR1513 were down regulated as indicated by the number of normalized sequences that mapped to the miRNA sequences (Table 4.4). The gma-miR169 targets CCAAT-binding transcription factor, gma-miR1508 is predicted to target the mRNA of a calcium-dependent protein kinase and gma-miR1513 is predicted to target mRNAs of F-box family proteins, galactose oxidase, starch synthase (Wong et al., 2011; Zhang et al., 2008). In signatures from leaves of ‘Mumford’ infected with viruses TSV and BPMV, gma-miR159a, gma-miR166, gma-miR390a-p were down-regulated whereas gma-miR396 was up-regulated (Table 4.5). MiR390a-p produces ta-siRNAs that regulate auxin response factors (Wong et al., 2011). In signatures from leaves of virus-infected ‘Williams 82’, gma-miRNA159a, gma-miRNA166r, isoform of gma-miR393, gma-miR396, gma-miR482*, gma-miR1507a, gma-miR2118 were down regulated and gma-

miR2118* and gma-miR390a-5p was up-regulated (Table 4.6). MiR393 targets the mRNAs of TIR1-like protein / F-box containing leucine rich repeat proteins (Subramanian et al., 2008), and gma-miR2118 is predicted to target mRNAs of a zinc finger transcription repressor/disease resistance protein (Wong et al., 2011).

Gene expression in virus-infected soybean

The changes in soybean transcript levels in virus-infected soybean tissues exhibiting symptom recovery, as determined by the numbers of sequence reads that aligned to predicted soybean transcripts, were largely dependent on the soybean cultivar. The mRNA-Seq analysis of the TSV- and BPMV-infected soybean cultivars ‘Mumford’ and ‘Williams 82’ generated 4.6 to 6.8 million sequence reads. The majority of the sequences generated by mRNA-Seq mapped to the soybean transcripts except in BPMV-infected ‘Williams 82’ (Table 4.7). The proportion of sequences that aligned to the soybean transcript in BPMV-infected ‘Williams 82’ accounted for only 14% and most mRNAs were down regulated except DCL2 (Glyma09g02930.1). From BPMV-infected ‘Williams 82’, 10 fpkm aligned to the predicted DCL2 transcript compared to 0 fpkm and 5 fpkm in mock-inoculated and TSV-infected ‘Williams 82’, respectively. DCL2 is involved in production of 22-nt siRNAs, which were the most abundant size class of small RNAs from BPMV-infected ‘Williams 82’. Glyma14g01900.1, which is predicted to encode an ATP-dependent glutathione S-conjugate transporter, had higher numbers of sequence reads in BPMV-infected ‘Williams 82’ at 6 fpkm compared to 1 fpkm and 4 fpkm in mock-inoculated and TSV-infected ‘Williams 82’, respectively. ATP-dependent glutathione S-conjugate transporter mediates detoxification by the uptake of glutathione S-conjugates into the vacuole (Marinoia et

al., 1993). The mRNA signature from BPMV-infected ‘Williams 82’ was very different from the other signatures and so was eliminated from further comparisons with other signatures.

Plant defense genes and stress response genes were affected by virus infection in both ‘Mumford’ and ‘Williams 82’. The numbers of sequences that aligned to predicted mRNA sequences from virus-infected soybean tissues with symptom recovery in both cultivars showed that osmotin (Glyma05g38130.1) and pathogenesis-related (PR) protein (Glyma06g12900.1) transcripts (Table 4.8) were up-regulated relative to mock-inoculated controls. Osmotin, a thaumatin-like PR protein, is also induced in tobacco infected by *Tobacco mosaic virus* (Stintzi et al., 1991). Transcripts for PAD4 (Glyma04g38700.1), a defense signaling protein also induced in other plants by infection with RNA viruses (Huang et al., 2005; Whitham et al., 2003), was up-regulated in virus-infected soybean. Increased expression of non-expressor of PR3 (NPR3, Glyma14g03510.1) and TGA3 transcription factor (Glyma13g02360.1), that can together regulated PR and defense-related gene expression (Fan and Dong, 2002; Kinkema et al., 2000), was observed in virus-infected soybean. Genes commonly induced by virus infections, such as aldo/keto reductase, calcium binding proteins, endochitinase, HSP70, thioredoxin and xyloglucan endotransglycosylase (Whitham et al., 2006), were also up-regulated in virus-infected soybean (Table 4.8). Whereas beta-1,3-glucanase 1 transcripts (BG1, Glyma03g28850.1), also a defense related gene (Bucher et al., 2001; Whitham et al., 2003), was up-regulated only in TSV-infected soybean and was down-regulated in BPMV-infected soybean. Virus infection of soybean increased the accumulation of mRNAs of stress related transcription factor RAP2.1 (Dong and Liu, 2010), and lowered expression some MYB (ATMYB16) and WRKY28 transcription factors. The accumulation of mRNAs for flavonoid

pathway related genes, GST and lactoylglutathione lyase (LGL), involved in methylglyoxal detoxification (Inoue and Kimura, 1995; Yadav et al., 2008) (Table 4.7) were down-regulated in virus-infected soybean.

TSV infection of ‘Williams 82’ increased the accumulation of mRNAs of stress-related transcription factor WRKY40 (Rushton et al., 2010), MYB (MYB14) and ethylene response factor (ERF) (Rushton and Somssich, 1998; Singh et al., 2002). Transcripts for proteins implicated in defense response such as mitogen-activated protein kinase (MAP) kinases, Non-Expressor of PR1 (NPR1) and phenylalanine ammonia-lyase (PAL) (Mishra et al., 2006; Singh et al., 2002) also accumulated at higher levels in ‘Williams 82’ infected with TSV as indicated by the number of sequence reads that mapped to the predicted transcripts. TSV infection in ‘Williams 82’ resulted in higher expression of some of the silencing related genes, but lowered expression of photosynthesis (chlorophyll, plastid and thylakoid) related genes (Table 4.8 and Fig 4.3). The proteins involved in signaling (kinases and chaperones), metabolism (energy, carbohydrate and lipid metabolism) and cellular (structural, basic cellular functions) were also influenced by TSV infection in ‘Williams 82’ (Fig. 4.3).

The mRNA-Seq analysis of virus infected tissues of ‘Mumford’ that exhibited symptom recovery revealed that the expression of stress-related transcription factors WRKY40 and MYB14, as indicated by the numbers of sequence reads that aligned to the predicted soybean transcripts, were down-regulated when compared to the mock inoculated plants (Table 4.8 and Fig. 4.4). Transcripts for the plant defense related protein, jasmonic acid carboxyl methyltransferase (Seo et al., 2001) and some disease resistance proteins were down-regulated in virus-infected soybean

cultivar ‘Mumford’. Fewer sequence reads aligned to silencing related genes in virus-infected ‘Mumford’ than mock-inoculated plants (Fig. 4.4). Transcripts of photosynthesis-related genes accumulated at a higher level in virus-infected ‘Mumford’ than mock-inoculated plants. Transcripts of proteins involved in metabolism and cellular functions were also down-regulated in virus-infected ‘Mumford’.

DISCUSSION

MiRNAs, *gma-miR159a* and *gma-miR166*, were down-regulated in virus-infected soybean cultivars ‘Itachi’, ‘Mumford’ and ‘Williams 82’ exhibiting symptom recovery. Both the miRNAs target mRNAs of plant defense response genes and down-regulation of the miRNA can increase accumulation of the target gene transcripts. The *miR159a* targets transcripts of GST (Zhang et al., 2008) and MYB family transcription factors (Song et al., 2011; Wong et al., 2011) and *gma-miR166* targets HD-Zip transcription factors. GST proteins play an important role in detoxification by binding to glutathione and attaching glutathione to electrophilic xenobiotics for vacuolar sequestration (Edwards et al., 2000). In *Arabidopsis*, GST is induced by hydrogen peroxide (Desikan et al., 1998), which is associated with initiation of programmed cell death in response to plant pathogens and also acts as a signaling molecule that can trigger plant defense responses by activating MAP-kinases (Hancock et al., 2002; Kovtun et al., 2000). GST has been implicated in resistance to fungal leaf diseases in maize and bacterial, fungal, and viral pathogens in rice (Wisser et al., 2005; Wisser et al., 2011). In *Arabidopsis*, programmed cell death associated with disease resistance rapidly and transiently induces MYB related gene in plants challenged by fungal and bacterial pathogens (Vailleau et al., 2002). MYB transcription factors

are also induced by abiotic stresses such as dehydration and salt stress conditions (Abe et al., 2003; Nagaoka and Takano, 2003). The down-regulation of gma-miR159 and gma-miR166 indicates that GST and MYB may also be involved in plant-virus interactions during symptom recovery.

The HD-Zip transcription factors, regulated by gma-miR166, participate in development and maintenance of organs and in plant responses to environmental conditions (Ariel et al., 2007). HD-Zip transcription factors have been linked to limiting programmed cell death in tomato and in plant responses to *Soybean mosaic virus* infection in soybean (Mayda et al., 1999; Wang et al., 2005). HD-Zip transcription factors, as indicated by the lower levels of gma-miR166, could be involved in disease recovery in BPMV- and TSV-infected soybean.

In soybeans infected with BPMV, the gma-miR168* strand accumulated at higher levels although proportional increases in miRNA accumulation were not observed. Suppression of mRNA targeting by inhibition of miRNA* strand degradation has been observed to be induced by the silencing suppressors expressed by TuMV, *Tomato bushy stunt virus* and *Beet yellow virus* (Chapman et al., 2004). The miR168 target AGO that is central to RNA silencing in antiviral plant defenses (Song et al., 2011). In plants infected with virus, AGO1 expression increases as a defense response against the virus, but with a decrease in AGO1 protein accumulation, which results from the induction of miR168 by virus silencing suppressors that repress translation of AGO1 mRNA (Varallyay et al., 2010). So, the accumulation of the miR168* can be a plant defense response against the up-regulation of miR168 by BPMV, although BPMV has no known silencing suppressor (Gu and Ghabrial, 2005). Even so, infection

of soybean plants by BPMV partially relieves silencing of chalcone synthase genes in maternal reproductive tissues (Hobbs et al., 2003) suggesting that BPMV expresses a RNA silencing suppressor that is active at least transiently in soybean.

In TSV-infected soybean tissues showing symptom recovery, isoforms of gma-miR398 and gma-miR398*, that target SOD gene transcripts (Jagadeeswaran et al., 2009), accumulated in higher levels. The SOD-targeting miR398 is down-regulated in *Arabidopsis* affected by abiotic stress or infiltrated by avirulent bacterial pathogens, which was suggested to be associated with the oxidative burst during hypersensitive resistance responses (Jagadeeswaran et al., 2009). In ‘Itachi’, TSV produced the most severe initial symptoms with extensive necrosis that were followed by symptom recovery. TSV-infected ‘Itachi’ also had higher levels of miR398 in recovered leaves than ‘Mumford’ or ‘Williams 82’. Hence as observed in *Arabidopsis*, the increased accumulation of miR398 might be associated with recovery after symptom development, which was most pronounced in TSV-infected ‘Itachi’.

RNA-Seq analysis revealed that the transcript levels of PR proteins, defense-related proteins and proteins commonly induced by RNA virus infection were regulated similarly in ‘Mumford’ and ‘Williams 82’ in tissues exhibiting symptom recovery from virus infection. PR and thaumatin-like proteins are generally expressed at higher levels in plants infected by RNA viruses (Whitham et al., 2003). Osmotin, a thaumatin-like protein PR protein, is also induced during hypersensitive reaction to TMV in tobacco (Stintzi et al., 1991). Induction of osmotin and PR proteins by BPMV and TSV in soybean might be a generalized plant response to virus infection. PAD4, NPR3 and TGA transcription factor proteins regulate salicylic acid synthesis during plant

defense responses (Shah, 2003; Vlot et al., 2009) and are induced by RNA virus infection (Whitham et al., 2003), were up regulated in virus-infected soybean relative to mock-inoculated controls. RNA virus infection associated proteins such as, aldo/keto reductase, calcium binding proteins, endochitinase, HSP70, thioredoxin, , and xyloglucan endotransglycosylase (Whitham et al., 2006) were also up-regulated in soybean infected with TSV and BPMV. Whereas other genes induced by virus infection in plants such as GST and flavonoid pathway genes (Gutha et al., 2010; Wisser et al., 2005; Wisser et al., 2011) accumulated at lower levels in TSV- and BPMV-infected plants than in controls. The abundance of transcripts for the methylglyoxal detoxifying enzyme LGL was down-regulated in virus-infected plants. In contrast, LGL mRNA abundance was enhanced during aphid feeding (Voelckel et al., 2004) and under salt stress (Sun et al., 2010) indicating that the encoded protein might be involved in responses to biotic and abiotic stress in plants.

The effect of virus infection followed by symptom recovery on most other genes was cultivar dependent. Genes with similar roles in defense response were affected in ‘Mumford’ and ‘Williams 82’, but had opposite effects on transcript levels. Plant defense-related transcription factors WRKY and MYB and disease resistance genes were down-regulated in symptom recovery exhibiting virus infected ‘Mumford’ and up-regulated in ‘Williams 82’. Transcripts of genes involved in ROS and detoxification (GST) were also down-regulated in ‘Mumford’ and up-regulated in ‘Williams 82’. And the transcripts of photosynthetic genes were up-regulated in ‘Mumford’ and down-regulated in ‘Williams 82’ with symptom recovery following virus infection. The cultivar specific response might be related to the difference in symptom severity as the initial symptoms on ‘Williams 82’ were much milder than in ‘Mumford’.

Transcripts of plant genes involved in RNA-silencing, such as DCL2, AGO4 and RDR1, were lower in virus-infected ‘Mumford’, but higher in virus-infected ‘Williams 82’ compared to mock-inoculated soybean. DCL1 transcript accumulation remained unchanged in all RNA samples, but DCL4 mRNA was up-regulated in TSV-infected ‘Williams 82’. DCL2 is important in the production of 22-nt virus derived small interfering (vsi) RNAs during plant defense responses to RNA virus infections and can compensate for DCL1 derived vsiRNA (Blevins et al., 2006; Deleris et al., 2006). DCL2 is also implicated in production of endogenous nat-siRNA (Borsani et al., 2005; Vaucheret, 2006). Nat-siRNAs are produced from natural antisense transcripts that have regions that are complementary to each other, one of which is expressed under stress conditions (Vaucheret, 2006). In the small RNA signatures from BPMV-infected ‘Williams 82’ the proportion of 22-nt small RNA were much higher than in mock-inoculated plants, which was also associated with higher expression of DCL2. However, in BPMV- and TSV-infected ‘Mumford’ and TSV-infected ‘Williams 82’ the levels of DCL2 did not affect the accumulation of 22-nt siRNA. This could indicate that DCL2 is involved in plant symptom recovery by affecting other pathways of small RNA generation (Borsani et al., 2005).

The AGO1 and AGO2 transcript accumulation levels did not differ significantly among the six treatments, whereas AGO5 expression was up-regulated in TSV-infected ‘Williams 82’ and AGO4 was affected differently in each of the soybean cultivars. The AGO4 plays an important role in transcriptional repression by RNA-directed DNA methylation (Zilberman et al., 2004). The AGO4 protein is directly involved in translational repression of virus RNAs and in resistance responses mediated by nucleotide-binding leucine-rich repeat resistance genes

(Bhattacharjee et al., 2009). The increased accumulation of AGO4 transcripts in ‘Williams 82’ and decreased accumulation in ‘Mumford’ might explain the suppression of initial virus symptoms in ‘Williams 82’ through translational repression of viral RNAs and the severity of initial symptoms in ‘Mumford’ in the absence of translational repression.

The levels of RDR transcripts also remained consistent among all RNA samples except for RDR1 in the RNA samples from virus-infected soybeans. In *Arabidopsis*, *Tobacco rattle virus*, TuMV and TMV vsRNAs production is dependent on RDR1 (Donaire et al., 2008; Garcia-Ruiz et al., 2010; Qi et al., 2009). The CMV silencing suppressor, 2b, inhibits RDR1 activity (Diaz-Pendon et al., 2007). Up-regulation of RDR1 in TSV-infected ‘Williams 82’ could also lead to virus suppression whereas in TSV-infected ‘Mumford’ RDR1 was down-regulated, which may be linked to increased initial symptom severity. Decrease in BPMV genomic RNA has been observed in plant part exhibiting symptom recovery in the soybean cultivar, ‘Jack’ (Lim et al., 2011). The cultivar dependent regulation of the plant defense-related genes and photosynthetic genes needs to be studied further. The role of GST, MYB transcription factor, HD-Zip transcription factor, and salicylic acid-mediated defense pathway in symptom recovery will need to be investigated.

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TABLES

Table 4.1. The number and proportion of small (s)RNA sequences of sizes 21 to 24 in the signatures from *Bean pod mottle virus* (BPMV)-infected, *Tobacco streak virus* (TSV)-infected and mock-inoculated soybean cultivars ‘Itachi’, ‘Mumford’, and ‘Williams 82’ that mapped to the soybean genome and soybean miRNA stem-loop sequences.

Signatures	sRNA of 21-24 nt	sRNA mapped <i>G. max</i> (21-24 nt)	% sRNA mapped <i>G. max</i> (21-24 nt)	sRNA mapped miRNA stem loop	% sRNA mapped miRNA stem loop
Itachi BPMV ^a	2468343 ^b	2254956 ^c	91% ^d	368052 ^e	16% ^f
Itachi Mock	2000115	1968668	98%	396358	20%
Itachi TSV	2797066	2159101	77%	244954	11%
Mumford BPMV	5305355	4879668	92%	1912869	39%
Mumford Mock	4400008	4345598	99%	2009496	46%
Mumford TSV	3606120	3191165	88%	1406502	44%
Williams 82 BPMV	22503758	20221786	90%	5571162	28%
Williams 82 Mock	5771766	5741626	99%	2988769	52%
Williams 82 TSV	13216859	12099988	92%	5889633	49%

^a Soybean cultivar ‘Itachi’ infected with BPMV

^b Small RNA of sizes 21 to 24 nt signature from BPMV infected ‘Itachi’

^c Small RNA of sizes 21 to 24 nt signature from BPMV infected ‘Itachi’ that mapped to the soybean genome

^d Percent small RNA of sizes 21 to 24 nt signature from BPMV infected ‘Itachi’ that mapped to the soybean genome

^e Small RNA of sizes 21 to 24 nt signature from BPMV infected ‘Itachi’ that mapped to the soybean miRNA stem loop from PMDB

^f Proportion (%) of small RNA that mapped to the soybean miRNA stem loop among the small RNAs that mapped to the soybean genome

Table 4.2. The miRNA, miRNA* and miRNA isoform from signatures of *Bean pod mottle virus* (BPMV)-infected soybean cultivars ‘Itachi’, ‘Mumford’ (Mf), and ‘Williams 82’ (W82) with similar changes when compared to mock-inoculated.

miRNA	miRNA sequence	Numbers of sequence hits per million reads (hpmr) ^a					
		Itachi BPMV	Itachi Mock	Mf BPMV	Mf Mock	W82 BPMV	W82 Mock
gma-miR1507b	UCUCAUCCAUCAUCG UCUG	45	48	224	102	558	206
gma-miR159a	UUUGGAUUGAAGGGAGC UCUA	35727	42539	9528	16700	3932	24843
gma-miR159a- isoform	UUUGGAUUGAAGGGAGC UCUU	73	87	53	159	22	141
gma-miR159d	AGCUGCUUAGCUAUGGA UCCC	86	32	279	54	1676	47
gma-miR166a/ gma-miR166b/ gma-miR166n/ gma-miR166o	UCGGACCAGGCUUCAU CCCC	4949	7619	197428	270878	134925	235724
gma-miR166b- isoform	UCUCGGACCAGGCUUCA UUCC	85	167	6566	10226	5231	12617
gma-miR166r	UCGGACCAGGCUUCAU CCCU	165	351	507	414	267	1461
gma-miR167c/ gma-miR167o	UGAAGCUGCCAGCAUGA UCUG	4789	6649	1235	2418	851	2858
gma-miR167e/ gma-miR167f	UGAAGCUGCCAGCAUGA UCUU	192	295	17	39	12	39
gma-miR168	UCGCUUGGUGCAGGUCG GGAA	193	175	716	397	887	429
gma-MIR168*	UCGCUUGGUGCAGGUCG GGAA	169	65	163	29	308	53
gma-miR168- isoform	UCGCUUGGUGCAGGUCG GGAAA	0	0	66	11	335	11

Table 4.2. continued

miRNA	miRNA sequence	Numbers of sequence hits per million reads (hpmr) ^a					
		Itachi BPMV	Itachi Mock	Mf BPMV	Mf Mock	W82 BPMV	W82 Mock
gma-miR396a	UUCCACAGCUUUCUUGA ACUG	4281	8489	181	50	29	326
gma-miR396b/ gma-miR396c	UUCCACAGCUUUCUUGA ACUU	2191	3178	3077	2485	2131	10756
gma-miR4345	UAAGACGGAACUUACAA AGAUU	0	0	5	3	16	5

^a The normalized to 1 million (hpmr) number of miRNA present in the signature from 'Itachi' infected with BPMV

Table 4.3. The miRNA, miRNA* and miRNA isoform from signatures of *Tobacco streak virus* (TSV)-infected soybean cultivars ‘Itachi’, ‘Mumford’(Mf), and ‘Williams 82’ (W82) that changed similarly when compared to mock-inoculated.

miRNA	miRNA sequence	Numbers of sequence hits per million reads (hpmr) ^a					
		Itachi TSV	Itachi Mock	Mf TSV	Mf Mock	W82 TSV	W82 Mock
gma-miR159a	UUUGGAUUGAAGGGAGCUC UA	18352	42539	8759	16700	10955	24843
gma-miR159a-isoform	UUUGGAUUGAAGGGAGCUC UU	34	87	68	159	74	141
gma-miR159a-isoform	UUUGGAUUGAAGGGAGCUC CA	20	45	20	46	32	55
gma-miR159a-isoform	UUUGGAUUGAAGGGCGCUC UA	15	58	6	9	6	13
gma-miR164	UGGAGAAGCAGGGCAGGUG CA	2753	5774	3	0	5	12
gma-miR396b / gma-miR396c	UUCCACAGCUUUCUUGAAC UU	1340	3178	3540	2485	5068	10756
gma-miR398a / gma-miR398b	UGUGUUCUCAGGUCACCCC UU	1216	25	0	0	0	0
gma-miR398a*	GGAGUGAAUCUGAGAACAC AAG	2288	16	0	0	0	0
gma-miR398b*	GGAGUGGAUCUGAGAACAC AAG	1080	204	4	0	3	0
gma-miR398-isoform	UUGUGUUCUCAGGUCACCCC CU	606	16	29	3	12	4
gma-miR398b*-isoform	GAGUGGAUCUGAGAACACA AGG	1920	170	47	6	19	3

^ahpmr: hits to miRNA normalized to 1 million mapped sequences.

Table 4.4. The miRNA sequences that were down-regulated in virus-infected ‘Itachi’ when compared to mock-inoculated ‘Itachi’

miRNA	miRNA sequence	Numbers of sequence hits per million reads (hpmr) ^a		
		BPMV ^b	TSV ^c	Mock
gma-miR159a	UUUGGAUUGAAGGGAGC UCUA	18352	35727	42539
gma-miR164	UGGAGAAGCAGGGCACG UGCA	3039	2753	5774
gma-miR166a / gma-miR166b / gma-miR166n / gma-miR166o	UCGGACCAGGCUUCAUU CCCC	4949	5668	7619
gma-miR169a / gma-miR169p	CAGCCAAGGAUGACUUG CCGG	354	109	653
gma-miR396a	UUCCACAGCUUUCUUGA ACUG	4281	3938	8489
gma-miR396b / gma-miR396c	UUCCACAGCUUUCUUGA ACUU	2191	1340	3178
gma-miR1508b- isoform	UAGAAAGGGAAAUAGCA GUUG	21113	9410	38775
gma-miR1508- isoform	CUAGAAAGGGAAAUAGC AGUUG	2528	716	3712
gma-miR1513- isoform	AAAGCCAUGACUUACAC ACGC	376	234	692

^ahpmr: hits to miRNA normalized to 1 million mapped sequences.

^bBPMV: *Bean pod mottle virus*

^cTSV: *Tobacco streak virus*

Table 4.5. The miRNA sequences that were down or up-regulated in virus-infected ‘Mumford’ when compared to mock-inoculated.

MiRNA	MiRNA sequences	Numbers of sequence hits per million reads (hpmr) ^a		
		BPMV ^b	TSV ^c	Mock
gma-miR159a	UUUGGAUUGAAGGGAGC UCUA	9528	8759	16700
gma-miR166a / gma-miR166b / gma-miR166n / gma-miR166o	UCGGACCAGGCUUCAUUC CCC	197428	188901	270878
gma-miR390a-5p	AAGCUCAGGAGGGAUAGC GCC	202	236	616
gma-miR396a	UUCCACAGCUUUCUUGAA CUG	181	106	50

^ahpmr: hits to miRNA normalized to 1 million mapped sequences.

^bBPMV: *Bean pod mottle virus*

^cTSV: *Tobacco streak virus*

Table 4.6. The miRNA sequences that were down or up-regulated in virus-infected ‘Williams 82’ when compared to mock-inoculated.

miRNA	miRNA sequences	Numbers of sequence hits per million reads (hpmr) ^a		
		BPMV ^b	TSV ^c	Mock
gma-miR159a	UUUGGAUUGAAGGGAGCUCUA	3932	10955	24843
gma-miR166r	UCGGACCAGGCUUCAUCCCU	267	736	1461
gma-miR167c / gma-miR167o	UGAAGCUGCCAGCAUGAUCUG	851	1865	2858
gma-miR2118	UUGCCGAUUCCACCAUCCUA	78	248	434
gma-miR393- isoform	UCCAAAGGGAUCGCAUUGAUCU	73	177	272
gma-miR396b / gma-miR396c	UUCCACAGCUUUCUUGAACUU	2131	5068	10756
gma-miR396a	UUCCACAGCUUUCUUGAACUG	29	151	326
gma-miR396- isoform	UUCCACAGCUUUCUUGAACUC	12	67	135
gma-miR482*	GGAAUGGGCUGAUUGGGAAGC	11025	16670	27726
gma-miR1507a	UCUCAUCCAUACAUCGUCUGA	46087	115651	154926
gma-miR2118*	GGAGAUGGGAGGGUCGGUAAAG	950	244	132
gma-miR390a-5p	AAGCUCAGGAGGGAUAGCGCC	0	0	130

^a hpmr: hits to miRNA normalized to 1 million mapped alignments

^bBPMV: *Bean pod mottle virus*

^cTSV: *Tobacco streak virus*

Table 4.7. The data generated from mRNA-Seq analysis from *Bean pod mottle virus* (BPMV)- and *Tobacco streak virus* (TSV)-infected and mock-inoculated soybean that aligned to the soybean transcripts and to the BPMV or TSV genomic RNA sequences.

mRNA signatures	mRNA-Seq signature size	Number and proportion sequences aligned to soybean transcript	Number and proportion sequences aligned to virus (BPMV/TSV) genomic sequences
Mumford BPMV	5,091,470	2,924,223 (57%)	1,032,944 (20%)
Mumford Mock	4,776,797	3,462,604 (72%)	892 (0%)
Mumford TSV	5,042,405	3,673,771 (73%)	4,771 (0%)
Williams 82 BPMV	4,667,980	649,758 (14%)	3,490,528 (75%)
Williams 82 Mock	5,526,769	4,127,720 (75%)	987 (0%)
Williams 82 TSV	6,831,120	5,085,994 (74%)	1028 (0%)

Table 4.8. The number of hits to some annotated soybean transcripts that are representative of soybean transcripts involved in photosynthesis and plant defense response in virus-infected and mock-inoculated ‘Mumford’ and ‘Williams 82’.

Soybean Transcript	Fragments per kilobase of exon per million fragments mapped (fpkm)					Annotations
	Mumford			Williams 82		
	BPMV	TSV	Mock	TSV	Mock	
Glyma05g38130.1	276	364	148	1153	95	ATOSM34 (osmotin 34) thaumatin family protein
Glyma06g12900.1	14	14	4	14	6	Pathogenesis-related family protein
Glyma04g38700.1	9	12	4	19	6	PAD4 (phytoalexin deficient 4)
Glyma03g28850.1	139	647	306	515	224	BG1 (beta-1,3-glucanase 1)
Glyma01g26230.1	14	19	36	7	15	Glutathione s- transferase
Glyma01g37360.1	71	73	192	40	69	Lactoylglutathione lyase family protein
Glyma11g07940.1	69	79	168	32	65	Lactoylglutathione lyase family protein
Glyma18g52470.1	2	2	1	3	1	HSP70 (heat shock protein 70)
Glyma16g10020.1	2	2	1	5	2	Disease resistance protein (TIR-NBS-LRR class)
Glyma03g14900.1	4	6	9	16	10	Disease resistance protein (TIR-NBS-LRR class)
Glyma17g07690.1	5	4	2	51	9	Nodulin family protein
Glyma14g03510.1	2	5	1	11	2	NPR3 (NPR1-LIKE PROTEIN 3)
Glyma13g02360.1	5	8	4	8	3	TGA3; calmodulin binding transcription factor
Glyma09g37600.1	19	10	4	10	4	Thioredoxin 2
Glyma15g36130.1	1	1	4	1	2	Ubiquitin-associated protein
Glyma08g08720.1	1	3	6	2	4	WRKY28 transcription factor
Glyma14g11920.1	4	14	30	41	8	WRKY40; transcription factor
Glyma13g01150.1	20	11	5	38	17	Xyloglucan endotransglycosylase 6
Glyma20g30480.1	23	18	55	19	42	Acidic endochitinase (CHIB1)
Glyma18g52250.1	3	4	13	6	12	Aldo/keto reductase, putative
Glyma14g37250.1	8	8	25	5	10	Calcium:cation antiporter
Glyma02g39150.1	13	19	43	8	20	Calcium:cation antiporter

Table 4.8. continued

Soybean Transcript	Fragments per kilobase of exon per million fragments mapped (fpkm)					Annotations
	Mumford			Williams 82		
	BPMV	TSV	Mock	TSV	Mock	
Glyma19g17230.1	6	6	17	2	5	Calcium exchanger
Glyma06g40330.1	37	39	17	64	19	Calcium-binding protein
Glyma12g34610.1	14	20	52	7	12	Calcium-binding protein
Glyma03g33660.1	1	1	4	3	6	MLO3, calmodulin binding
Glyma16g08900.1	1	6	10	24	5	MLO1; calmodulin binding
Glyma18g49630.1	4	5	9	2	5	ATMYB16, MYB factor
Glyma12g11390.1	14	23	49	109	49	MYB14 transcription factor
Glyma14g09320.1	24	14	7	44	24	RAP2.1 (related to AP2 1)
Glyma11g01350.1	8	10	22	4	9	Naringenin-chalcone synthase
Glyma19g27930.1	163	233	415	107	262	Naringenin-chalcone synthase
Glyma06g21920.1	9	14	43	15	31	Flavonoid monooxygenase
Glyma11g32520.1	18	24	4	33	12	Protein kinase family protein
Glyma17g32780.1	3	5	1	59	13	Protein kinase family protein
Glyma13g44640.1	2	3	6	2	7	Protein kinase family protein
Glyma07g07650.1	3	4	1	14	4	Protein kinase family protein
Glyma03g22230.1	9	14	21	48	23	Oxidative signal kinase
Glyma02g06070.1	4	13	67	25	4	Jasmonic acid carboxyl methyltransferase
Glyma09g02930.1	1	4	5	5	0	DCL2 (Dicer-like 2)
Glyma14g04510.1	5	9	13	7	5	AGO4; Argonaute 4
Glyma02g09470.1	9	19	23	25	8	RDR1; RNA-dependent RNA polymerase 1
Glyma13g07610.1	4650	4803	2207	2493	5155	RuBisCO small subunit 3B
Glyma18g14410.1	402	376	195	264	566	Photosystem II subunit P-1
Glyma16g33030.1	641	476	311	340	662	Light harvesting complex of photosystem II

FIGURES

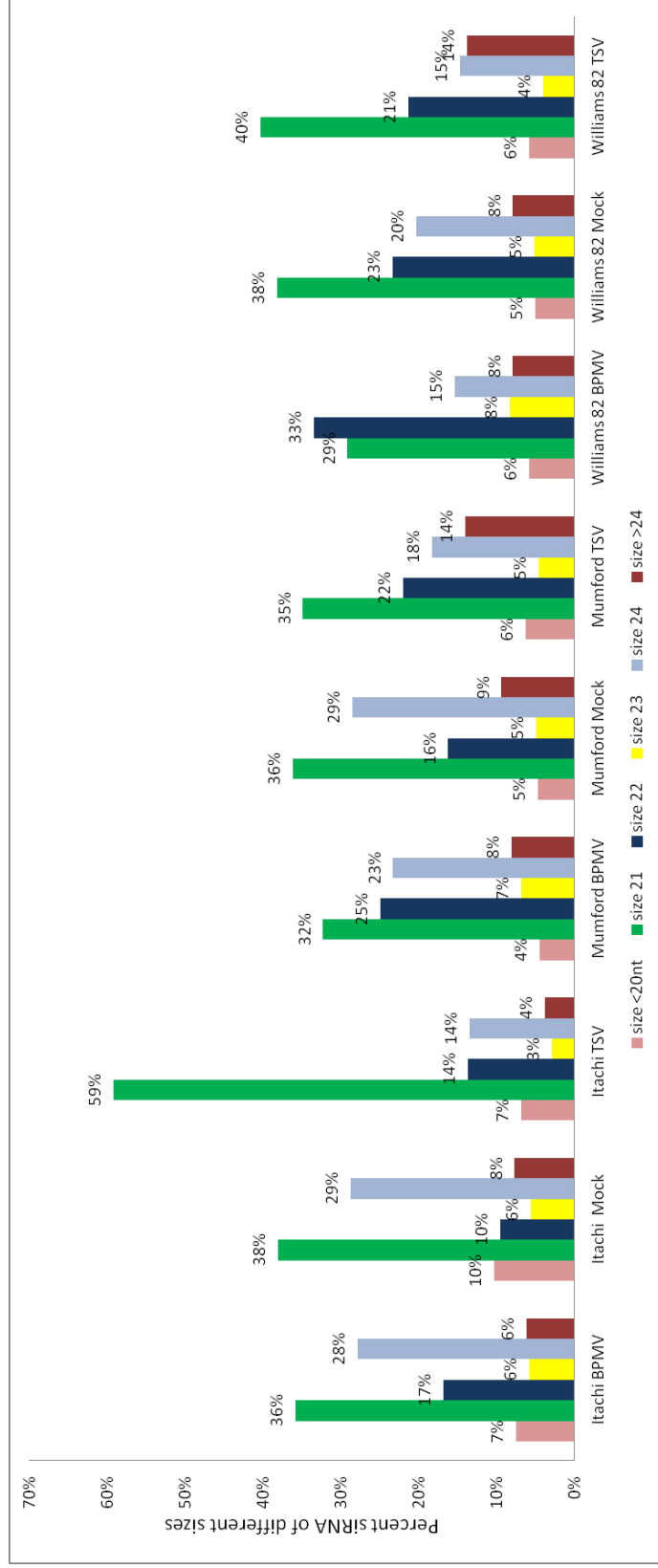


Figure 4.1. The proportion of small RNA of sizes 16-20 nt (pink), 21-nt (green) 22-nt (dark blue), 23-nt (yellow) and 24-nt (light blue) and 25-30 nt (maroon) in the signatures from soybean cultivars, ‘Itachi’, ‘Mumford’ and ‘Williams 82’, infected with viruses BPMV or TSV and mock inoculated.

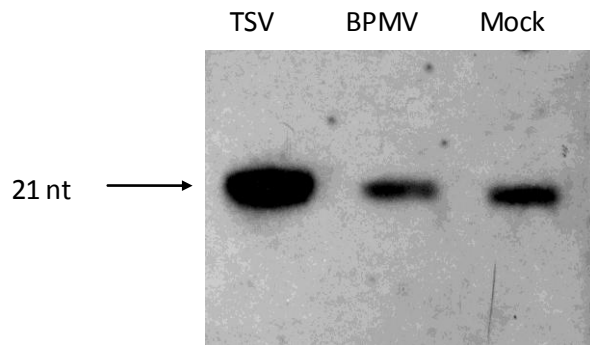
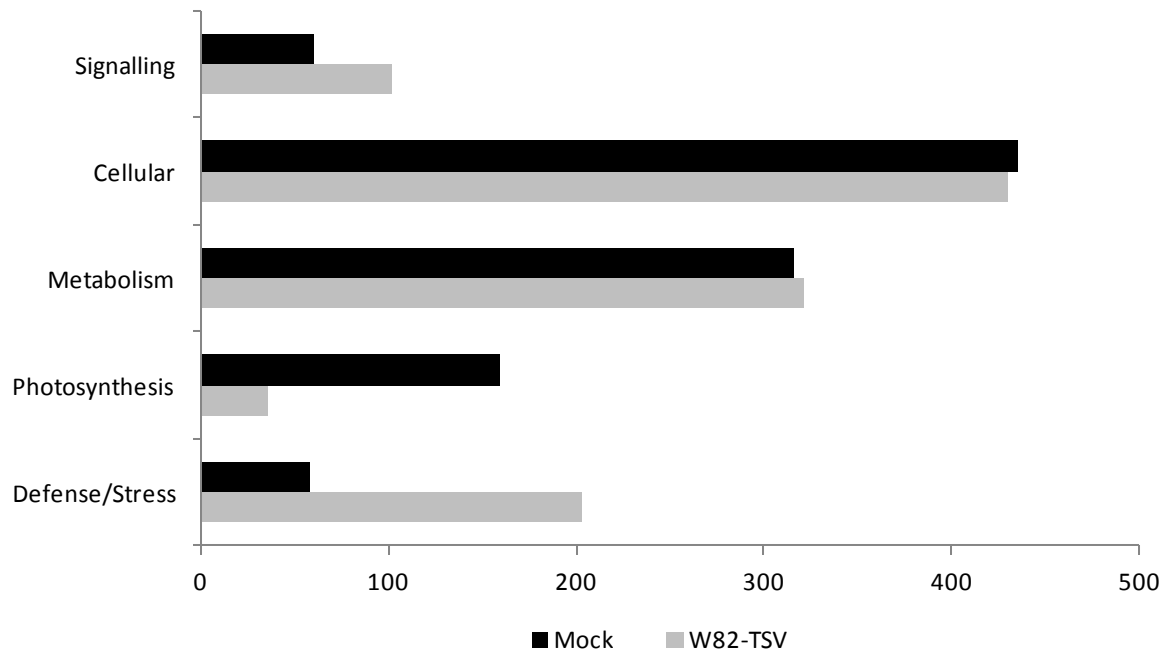


Figure 4.2. Small RNA northern blot of miRNA398 from 'Itachi' infected with *Tobacco streak virus* (TSV) or *Bean pod mottle virus* (BPMV) and mock inoculated plants.

A



B

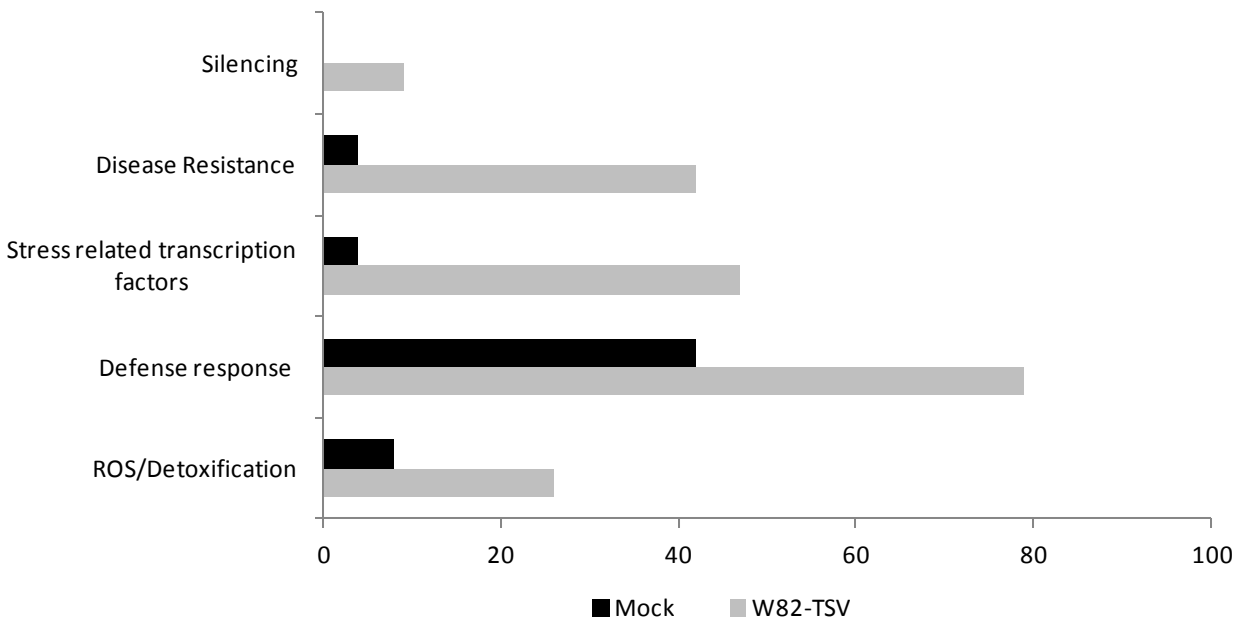
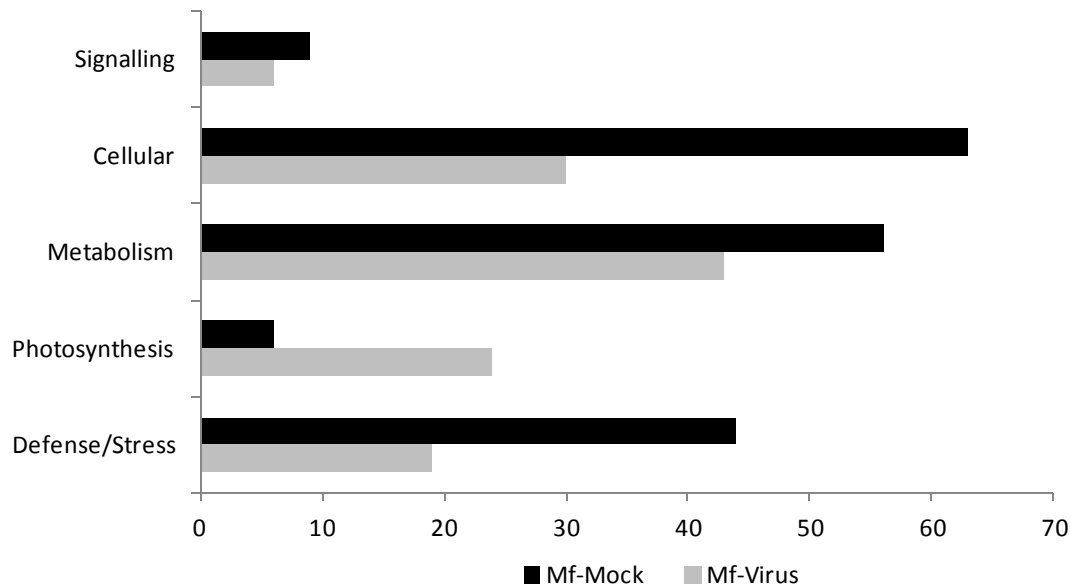


Figure 4.3. The transcripts of annotated genes expressed in higher levels in mock-inoculated (black bars) and *Tobacco streak virus* (TSV)-infected ‘Williams 82’ (W82-TSV, blue bars) A. Categorized based on functions of signalling, cellular, metabolism, photosynthesis and plant defense. B. Defense related genes categorized based on role in silencing, disease resistance, stress (transcription factors), defense response, and reactive oxygen species (ROS) homeostasis and detoxification-related proteins.

A



B

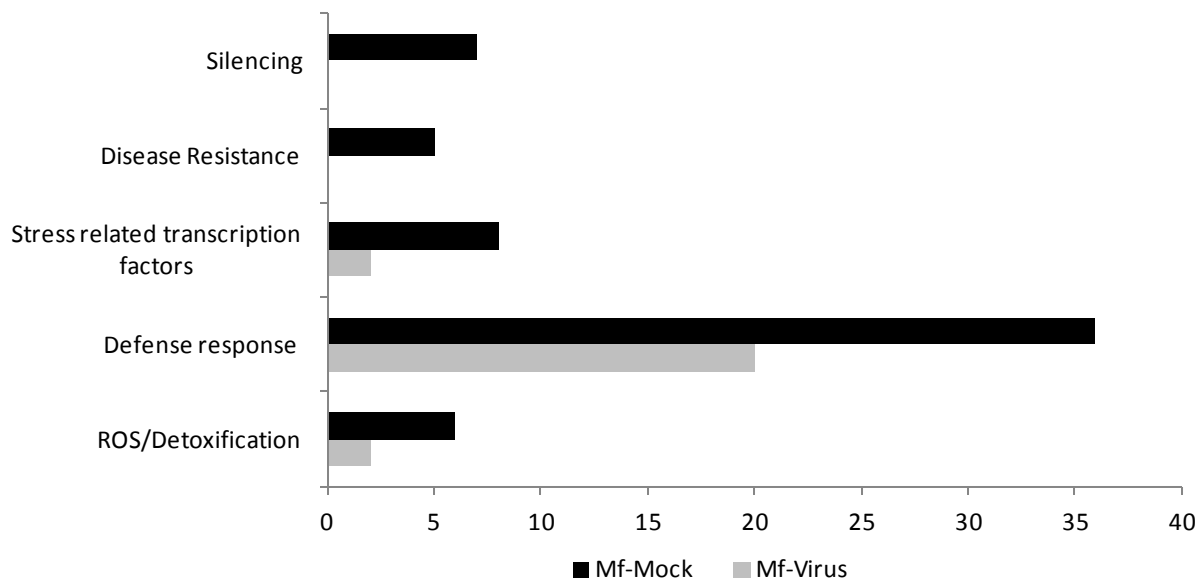


Figure 4.4. The transcripts expressed at higher levels in mock-inoculated (black bars) and virus infected ‘Mumford’ (Mf-Virus, gray bars) with either *Bean pod mottle virus* (BPMV) or *Tobacco streak virus* (TSV) A. Categorized based on functions of signalling, cellular, metabolism, photosynthesis and plant defense. B. Defense related genes categorized based on role in silencing, disease resistance, stress (transcription factors), defense response and reactive oxygen species (ROS) homeostasis and detoxification related proteins.