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Characterization of Seed Transmission of Soybean Mosaic Virus in Soybean

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

CHARACTERIZATION OF SEED TRANSMISSION OF *SOYBEAN MOSAIC VIRUS* IN SOYBEAN

(Thesis format: Monograph)

by

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ABSTRACT

Infection by *Soybean mosaic virus* (SMV) is recognized as a serious, long-standing threat in most soybean (*Glyince max* (L.)Merr.) producing areas of the world. The aim of this work was to understand how SMV transmits from infected soybean maternal tissues to the next generation by investigating the possible routes and amounts of seed transmission of SMV. Analysis of seeds at various reproductive stages revealed that SMV infects all parts of the seed, including the embryo, cotyledon and testa. *In situ* hybridization and immunofluorescence studies detected the presence of negative sense RNA and dsRNA in the suspensor base regions and embryonic tissues. Ultrastructural studies revealed the hallmark pinwheel aggregates of cylindrical inclusions in the infected cells of leaves and seed embryos. Up to 26% of SMV-positive seeds were recorded from the infected Williams 82 cultivars. Taken together, these results suggest that the seed embryo is a potent source for SMV transmission.

Key words: SMV, Seed transmission, embryo, suspensor

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LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	Complimentary DNA
C-Terminal	Carbon Terminal
CI	Cylindrical Inclusion
CP	Coat Protein
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DEPC	Diethylpyrocarbonate
dpi	Days post inoculation
ds	Double stranded
EDTA	Ethylene diamine tetra acetic acid
eIF4E	Eukaryotic translation initiation factor 4E
ELISA	Enzyme linked immunosorbent assay
g	Gram
GTP	Guanosine triphosphate
HCl	Hydrogen chloride
HC-Pro	Helper component protease

″	Inches
ISH	In Situ Hybridization
kDa	Kilodalton
lb	Pound
M	Molar
mM	millimolar
mg	Milligram
µM	Micromole
µL	Microlitre
min	Minute
miRNAs	micro RNAs
mg	Milligramme
mL	Millilitre
mRNA	Messenger RNA
nm	Nanomètre
nt	Nucleotide
ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
%	Percentage
Poly A	Polyadenylation A
PTGS	Posttranscriptional gene silencing
qRT-PCR	Reverse Transcription qPCR

RISC	RNA-induced silencing complex
rRNA	Ribosomal RNA
RNAi	RNA interference
RNA	Ribonucleic acid
rpm	Round per minute
RT	Room Temperature
s	Second
SCPFRC	Southern crop protection and Food research center
SDS	Sodium Dodecyl Sulphate
SM	Soybean Mosaic
SMV	Soybean Mosaic Virus
TAE	Tris-acetate EDTA
TBE	Tris/Borate/EDTA Buffer
TEM	Transmission Electron Microscopy
TF	Transcription factor
tRNA	Transfer RNA
Tris	Tris-(hydroxylmethyl)-methylamine
2,4-D	2,4-Dichlorophenoxyacetic acid
U	Unit
UTR	Untranslated region
UV	Ultra-violet
Vol	Volume
w/v	Weight/Volume

1 INTRODUCTION AND LITERATURE REVEIW

1.1 Overview

Soybean (*Glycine max* (L.) Merr.) is an annual legume that serves as one of the principal food sources for humans and livestock. Soybean oil is one of the most common vegetable oils and is considered to be an important future bio-diesel (fuel). Soybean is an important oil seed, averaging approximately 43% protein and 20% oil by weight (Carrera *et al*., 2011). As one of the top seven soybean producing countries in the world, Canada produces nearly 5 x 10^6 tons of soybeans per year (Government of Canada, Statistics Canada, 2014) most of which is grown in Southern Ontario and Québec. Soybean is affected by various biotic and abiotic stresses. Among pathogens, there are a total of 67 viruses that infect soybean and 27 of them are considered a threat to the industry (Tolin and Lacy, 2004; Maroof *et al.*, 2008). *Soybean mosaic virus* (SMV) is the most widespread virus and is recognized as the most notorious and long-standing problem in many soybean producing areas (Wang, 2009). It has been documented that the yield losses usually range from 8 to 50% under natural field conditions (Hill, 1999; Arif and Hassan, 2002) and reach up to 100% in severe outbreaks (Liao *et al.*, 2002). Mixed infections by SMV and one or more other viruses such as *Bean pod mottle virus* (BPMV), *Alfalfa mosaic virus* (AMV) and *Tobacco ringspot virus* (TRSV) often cause far more severe damage than infection by single virus (Hill *et al*., 2007; Wang, 2009). Importantly, SMV can be transmitted from infected soybean maternal tissues to the next generation via seeds, although the mechanism remains unknown.

1.2 Literature Review

1.2.1 General, Biological and Physical Properties of SMV

In 1916, Clinton reported Soybean mosaic (SM) disease, but the details of the disease were not described until 1921 by Gardner and Kendrick. Since then, the virus has been found in China, Japan, South Korea, Canada, United States of America, Brazil, Australia and many other countries where soybean is grown. Soybean mosaic diseased plants were reported to show stunted stature, and distorted leaflets containing mosaic dark green areas, while seed transmission and mechanical inoculation were suggested to be important routes for SM disease spread (Gardner and Kendrick, 1921). Now it is known that SM disease is caused by SMV, a member of the genus *Potyvirus* of the family *Potyviridae*, and that it is one of the most widespread diseases of soybean. Early infection of a soybean plant can result in severe effects such as reduced pod set, seed size, poor oil content of the seed and root nodulation (Hill, 1999). SMV infection also causes reduce seedling viability, seed quality and result in poor seedling vigor and seed coat mottling (El-Amretz *et al*., 1987b; Hobbs *et al*., 2003).

Potyviruses represent the largest group of known plant viruses and includes more than 150 members causing significant losses in a wide range of crop plants (Fauquet and Mayo, 1999). SMV viral particles are flexuous rod-shaped, 650-740 nm in length and 15 to 18 nm in diameter (ICTVdB Management, 2006). Encapsidated viral particles contain the viral genome of a linear, positive-sense, single-stranded RNA molecule. The thermal inactivation point of the particle is usually 10 min at 55-60 ^{o}C. When plant sap is stored at 4 ^{o}C, the shelflife of the virus *in vitro* is 14 to 15 days and the dilution end point is usually around 10^{-3} to 10^{-5} (Hill, 1999). However, SMV is most stable at pH 6 in

expressed sap and loses infectivity at pH levels below 4 and above 9 (Galvez, 1963). CI protein is one of the major proteins of SMV, and these produce cylindrical, pinwheel-shaped inclusions that are a characteristic cellular phenotype for potyvirus infection and are often found in the cytoplasm of infected cells.

1.2.2 Host Range and Symptoms

SMV is known to naturally infect the relatives of soybean such as *Fabaceae* (also *Leguminosa*), *Amaranthaceae, Chenopodiaceae, Passifloraceae, Schropulariaceae and Solanaceae* (Bos, 1972; Sinclair, 1982); however, its most common hosts are plants in the *Fabaceae* (Galvez, 1963; Hill, 1999). SMV has also been isolated from naturally infected *Cassia occidentalis* in Nigeria and *from Vicia faba* showing mild symptoms of yellow mottle in China (Thottappilly, 1985).

SMV-induced symptoms depend on several parameters such as virus strain, host genotype, plant age at the time of infection, and environment conditions. Rugosity, stunting, leaf curling, dark green vein banding, light green interveinal areas, seed coat mottling, flower deformation, necrosis, sometimes necrotic local lesions, systemic necrosis and bud blight are commonly observed in SMV-infected soybean plants (ICTVdB Management, 2006). Some of these SMV symptoms may be masked at temperatures above 30 °C (Hill, 1999).

1.2.3 Strains

To date, a large number of SMV isolates have been reported in the world. Isolates of SMV have been classified into categories G1-G7 based on their ability to break resistance genes (Cho and Goodman, 1979). Later, two more groups, G7A and C14 were added (Buzzell and Tu, 1984; Lim, 1985). Similarly, five (A to E) and eight (Sa to Sh) additional SMV strains have been reported in Japan and China, respectively (Takahashi *et al*., 1980; Pu *et al*., 1982; Chen *et al*., 1986). Recently, several SMV isolates have also been identified in Canada: a necrotic strain, SMV-N, and a G2 isolate (Gagarinova *et al*., 2008a; Farsi *et al*., 2009; Wang, 2009). But due to high sequence similarity of SMV-N and G2, SMV-N is thus considered a G2 isolate (Gagarinova *et al*., 2008b).

1.2.4 SMV-host interactions

Due to the availability of numerous soybean cultivars and a large number of SMV isolates, the interaction between SMV and soybean plant may be among the most complex virus-plant interactions known. As briefly mentioned above, SMV is classified into different strains, based on their different responses on several susceptible and resistant cultivars. To date, three independent loci for SMV resistance, *Rsv1*, *Rsv3* and *Rsv4*, that confer resistance to SMV with strain specificities, have been identified in the soybean germplasm (Hayes *et al.*, 2000; Zheng *et al.*, 2005).

Rsv1 is a dominant gene of soybean conferring resistance to SMV strains G1-G6 but not to G7, whereas *Rsv3* gives resistance to G5-G7 but is susceptible to G1-G4. *Rsv4* is the only resistance gene that confers resistance to all seven G-strains (Hayes *et al.*, 2000;

Gunduz *et al*., 2001; Zheng *et al.*, 2005), but only conditions resistance at the seedling stage and exhibits a delayed susceptible phenotype at the later growth stage. *Rsv1* was mapped to the molecular linkage group F (soybean chromosome 13) in a cluster of resistance genes (Gore *et al*., 2002; Hwang *et al*., 2006). *Rsv3* was mapped to the molecular linkage group B2 (soybean chromosome 14), also containing a cluster of disease resistance genes (Jeong *et al*., 2002). *Rsv4* was mapped to the molecular linkage group D1b (soybean chromosome 2) which doesn't contain any other resistance genes (Hayes *et al*., 2000; Maroof *et al*., 2010).

These data suggest a high level of complexity for SMV-soybean interactions and the limitation of resistance genes. In addition, resistance-breaking isolates overcoming all the known resistance loci have been reported in major soybean producing countries such as Canada, The United States, South Korea and Brazil (Almeida *et al*.,1995; Hajimorad *et al*., 2003; Choi *et al*., 2005; Gagarinova *et al*., 2008a). The occurrence of resistance-breaking isolates suggests a high risk associated with the utilization of these three resistance genes. Novel strategies against SMV are in high demand.

1.2.5 SMV genome and gene functions

Like other potyviruses, SMV has a single stranded positive-sense RNA molecule of approximately 9.6 kb in length as its genome (Adams *et al*., 2005; Gagarinova *et al.*, 2008a). The genomic RNA of the virus is packaged in the protein shell made of multiple copies of coat protein (CP) subunits. The genomic RNA has a viral genome-linked protein (VPg) covalently linked to the 5' end and a polyadenylate (PolyA) tail at the 3'

end. The genome also contains 5' and 3' non-translated regions (NTRs) (Reichmann *et al*., 1992).

The potyviral genome contains a large open reading frame (ORF) plus a smaller ORF that results from translational slippage in the large ORF (Figure 1) (Jayaram *et al*., 1992; Urcuqui-Inchima *et al*., 2001; Chung *et al*., 2008; Gagarinova *et al*., 2008a). The two polyproteins encoded by these two ORFs are co and post-translationally processed into 11 final products (Figure 1) by 3 viral proteases (P1, HC-Pro and N1a-Pro). The 11 mature proteins beginning from the N terminus of the polyprotein are: P1 (the first protein), HC-Pro (the helper component/protease), P3 (the third protein), P3N-PIPO (resulting from the frame-shift in the P3 cistron), 6K1 (the first 6kDa peptide), CI (the cylindrical inclusion protein), 6K2 (the second 6 kDa peptide), NIa-VPg (nuclear inclusion "a"–viral genome-linked protein; also VPg), NIa-Pro (nuclear inclusion "a" protein–the protease), NIb (the nuclear inclusion "b" protein) and CP (coat protein) (Jayaram *et al*., 1992). Most of these viral proteins are multi-functional (Jayaram *et al*., 1992; Chung *et al*., 2008; Gagarinova *et al*., 2008a).

The P1 protein is the most variable among potyviruses, ranging in size from 30 kDa to 63 kDa (Domier *et al*., 1987; Verchot and Carrington, 1995a). It is a serine protease that automatically cleaves the polyprotein at the C-terminal of P1 (Urcuqui-Inchima *et al*., 2001). P1 is an accessory factor that can enhance viral amplification and movement (Verchot and Carrignton, 1995b). Mutation of the cleavage site at the P1/HC-Pro boundary renders the virus nonviable.

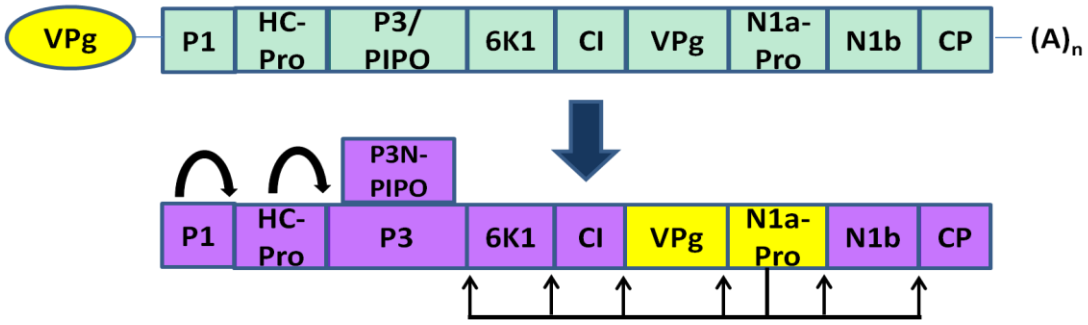

Figure 1: **Schematic representation of the genome of potyviral SMV.** The SMV genome is translated into a single polyprotein which is subsequently processed by virus-encoded proteases into first protein (P1), helper component proteinase (HC-Pro), third protein (P3), cylindrical inclusion (CI) protein, 6K (6 kDa), viral genome-linked protein (VPg), nuclear inclusion proteins (NIa and NIb) and coat protein (CP). The P3N-PIPO protein is produced from a separate small ORF. Proteolytic sites are marked with arrows indicating the names of the corresponding proteases. Viral RNA is shown in light green and translated proteins in light purple color (Adams *et al*., 2005; Ng and Falk, 2006, Ivanov *et al*., 2014).

Along with HC-Pro, the P1 protein is also a silencing suppressor and has been suggested to play a pivotal role in virus host range (Anandalakshmi *et al*., 1998; Rajamaki *et al*., 2005; Salvador *et al*., 2008; Valli *et al*., 2006). Aside from its protease activities, P1 also shows RNA binding activity, although the exact function is still unknown (Urcuqui-Inchima *et al*., 2001). HC-Pro is an interesting and well-studied multifunctional protein (Syller, 2006). In addition to other activities, HC-Pro 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). HC-Pro has also been found to serve diversified functions in potyviruses such as local and systemic movement, genome amplification, host gene silencing suppression and symptom development (Urcuqui-Inchima *et al*., 2001; Syller, 2006). Most importantly, this protein acts as a bridge between viral particles and the aphid food canal in the stylet, where it mediates the uptake and release of viral particles, allowing plant to plant transmission of the virus (Pirone and White, 1996; Peng *et al*., 1998). The central region of HC-Pro affects long distance movement, genome amplification and gene silencing suppression (Syller, 2006). The C terminal region of HC-Pro is involved in cell to cell movement (Rojas *et al*., 1997) and it also contains a cysteine-type protease motif (Syller, 2006). HC-Pro also has been found to increase the plasmodesmatal size exclusion limit; facilitating the transmission of viral RNA between cells (Rojas *et al*., 1997). The molecular weight (MW) of this protein in SMV is 51 kDa.

A recent study showed that seed transmission of SMV is influenced by P1, HC-Pro, and CP proteins (Jossey *et al*., 2013). Another recent study revealed a previously unknown function of HC-pro, i.e., enhancing the stability of its cognate CP to increase the yield of

virions and consequently improving the infectivity of the viral progeny (Valli *et al*., 2014).

P3 is a membrane-associated protein that binds to the Golgi apparatus and endoplasmic reticulum (ER) in the cell and moves along actin microfilaments and thus may play a role in virus replication and intracellular movement (Cui *et al*., 2010; Eaitanaste *et al*., 2007). P3 has been shown to act as an elicitor for *Rsv1* and *Rsv4*-mediated resistance in soybean plants (Hajimorad *et al*., 2008; Chowda-Reddy *et al*., 2011 and Wang Y *et al*., 2014). The MW of P3 protein of SMV is around 42 kDa.

P3N-PIPO (the N terminus of P3 and the pretty interesting potyviral ORF-encoded protein) is a recently discovered 25-kDa protein. The protein is produced by a translational frameshift near the middle of the P3 cistron (Chung et al, 2008). P3N-PIPO forms a complex with the CI protein that can interact with the plasmodesmata to facilitate viral cell-to-cell virus movement (Wei *et al*., 2010a).

The CI protein, also known as ATP-dependent RNA helicase, plays a role in virus replication and viral intercellular spread (Lain *et al*., 1990). The CI protein is the major component of characteristic cylindrical inclusions structure that accumulates in the cytoplasm of the infected cell (Dougherty and Hiebert, 1980) and has been proposed to play a direct role in potyviral replication. The CI protein of SMV is 71 kDa in size.

There are two 6K proteins. The 6K2 protein is another membrane bound protein that can induce the formation of membranous vesicles derived from the ER and trafficking to the chloroplasts, and these 6K2 vesicles serve as sites for virus replication or RNA translation (Cotton *et al*., 2009; Wei *et al*., 2010). The actual role of 6K1 is not clear so

far but it has been suggested to be involved in viral cell-to-cell movement. The lack of transmembrane domains in 6K1 makes it a poor candidate as a typical viral movement protein (Hong *et al*., 2007). However, P3 and 6K1 are normally found associated together and play an essential role in plant pathogenicity (Urcuqui-Inchima *et al*., 2001).

N1a has two parts: the N terminal VPg and the C-terminal trypsin-like serine protease. The latter acts *in cis* and *in trans* cleaving the polyprotein at all the cleavage sites except the two sites processed by P1 and HC-Pro. The VPg, as mentioned earlier, binds to the 5'-termini of genomic RNAs and interacts with the eukaryotic translation initiation factor, eIF4E, and the viral protein HC-Pro for virus RNA translation (Puustinen and Makinen, 2004; Roudet-Tavert *et al*., 2007). The VPg protein is a 22 to 24 kDa protein (Siaw *et al*., 1985; Shahabuddin *et al*., 1988; Riechmann *et al*., 1989; Murphy *et al*., 1990; Laliberte *et al*., 1992) in other potyviruses, and relatively larger (about 27 kDa) in SMV. Potyviral VPg has also been suggested to function as a sense-mediated RNA silencing suppressor (Rajamaki *et al*., 2014). The NIb is the RNA-dependent RNA polymerase that is responsible for catalysis of viral genome multiplication (Domier *et al*., 1987).

CP is involved in virus assembly, cell to cell movement and aphid transmission of the virus (Urcuqui-Inchima *et al*., 2001). CP was also found to have a stimulatory role in genome amplification (Mahajan *et al*., 1996). A recent study showed that aphid and seed transmission of SMV was influenced by CP along with P1 and HC-Pro (Jossey *et al*., 2013). The MW of the potyvirus CPs ranges from 28 to 40 kDa.

1.2.6 SMV replication

SMV-soybean interaction is a compatible reaction in susceptible cultivars. In addition to seed transmission, SMV enters soybean cells either through a mechanical abrasion or by an aphid vector. Subsequent uncoating of the viral RNA takes place in the cytoplasm followed by genome translation and replication (Figure 2). Such compatible virus infection often induces and suppresses host gene expression at the global level (Whitham *et al*., 2006). Accumulated evidence suggests that viral replication is associated with intracellular membranous structures (Wei and Wang, 2008), which form vesicles near membranes that have been proposed to provide a scaffold for anchoring the virus replication complex (VRC). The VRC conditions the RNA replication process in a specific safeguarded cytoplasmic location to prevent the activation of host defense responses, and to recruit the viral and host components required for replication and maintain the proper concentrations of these components (Wileman, 2006). The Potyviral VRC-containing vesicles also seem to originate at the endoplasmic reticulum exit sites (ERES), traffic along the microfilaments and target chloroplasts for replication (Wei *et al*., 2010b). Viral vesicles are also transported along actin filaments towards the cell wall and PD. At least four viral proteins, CI, VPg, CP and P3N-PIPO are thought to take part in viral cell-to-cell movement through PD, which involves the formation of PD-associated pinwheel structures (Figure 2). For replication, a number of host proteins are recruited by the virus at the vesicular compartments such as heat shock cognate 70-3 (Hsc70-3), poly (A)-binding protein (PABP), eEF1A and eIF(iso)4E along with several non-structural viral proteins, such as the 6K2, NIb (the viral RNA dependent RNA polymerase, RdRp), NIa (including NIa-VPg or NIa-Pro or as a precursor protein) and CI

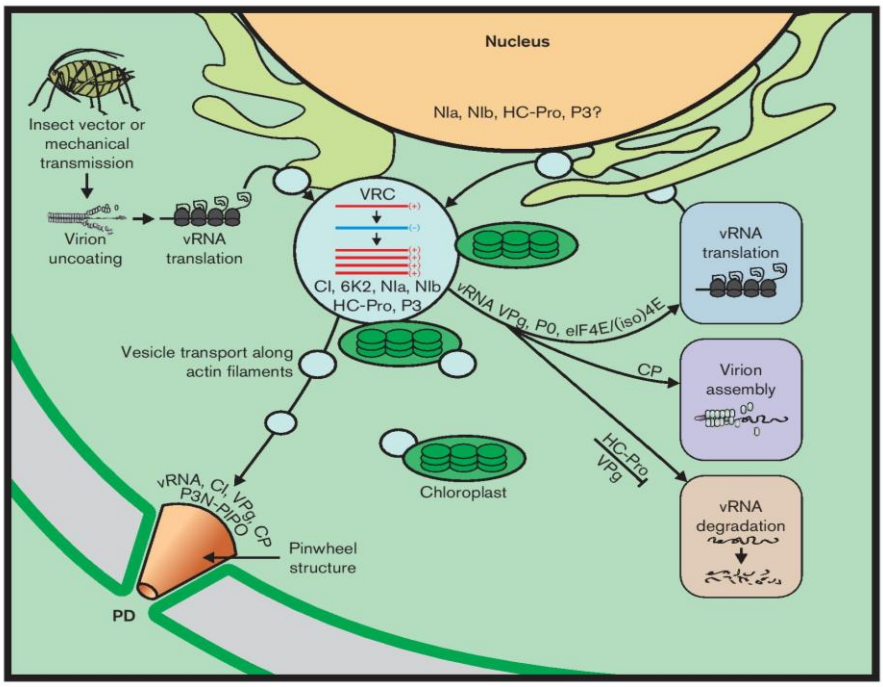

Figure 2: Schematic representation of major events in the potyvirus infection cycle. Through the stylet of an aphid vector or by mechanical inoculation filamentous virions enter the host cell. Following virion uncoating in the cytoplasm, genomic RNA is translated into a polyprotein that is subsequently processed by three viral proteases. CI, 6K2, NIa, NIb, HC-Pro and P3 are the six viral proteins played a role in viral replication. Membranous vesicles at ERESs or chloroplast are used as replication complexes (VRCs) for potyvirus. Viral vesicles are transported along actin filaments towards the cell wall and PD. Viral cell-to-cell movement occurred by four viral proteins, CI, VPg, CP and P3N-PIPO, which involves the formation of PD-associated pinwheel structures. Alternatively, viral vesicles are transported directly into neighbouring cells through the PD. Viral RNA originated into the VRC can be translated and incorporated into progeny VRCs, and readily turned into new rounds of viral replication. Alternatively, viral RNA can be packaged into virions (Ivanov *et al.*, 2014).This picture is used by the permission of copyright holder (Appendix A).

(Dufresne *et al*., 2008; Thivierge *et al*., 2008; Cotton *et al*., 2009; Wei *et al*., 2010; Ivanov *et al*., 2014).

Within the induced vesicles, the RdRp binds to the 3' termini of the viral (+) RNA to initiate transcription of negative strand replicative RNA. This negative sense (-) RNA intermediate is used as a template to produce progeny (+) RNAs that are delivered to the cytoplasm for translation or encapsidation. The CP binds positive-sense progeny (+) RNAs to form progeny virions with VPg attaching at the end. The potyviral replication possibly begins with uridylylation of VPg which acts as a primer for progeny RNA synthesis, a process shared by the *Picornaviridae* family (Puustinen and Mäkinen, 2004).

1.2.7 SMV movement

Once viral infection has occurred, there are two types of viral movement throughout the soybean. Viral movement can be either intercellularly through the plasmodesmata or systemically, where it can move through the sieve elements to infect more distant parts of the host. Recent studies have shown that the proteins, VPg, CP, HC-Pro, CI and P3N-PIPO are important for intercellular movement (Cronin *et al*., 1995; Wei *et al*., 2010). VPg is attached to the 5’ end of the viral RNA and serves as a signal to the plasmodesmata, indicating where HC-Pro and CP may interact with the plasmodesmata so that the virion can travel through. The CI protein creates a cone-shaped structure at plasmodesmatal openings with the help of P3N- PIPO. This allows the virion to associate with and pass through the channel and into the next cell (Carrington *et al*., 1998; Wei *et*

al., 2010). The CI protein is an essential factor in cell-cell movement and mutations in the gene restrict the virus to the single primarily infected cell (Carrington *et al.*, 1998).

In order for systemic infection to occur in the plant, SMV must move to the distal parts of the plant. For long-distance or phloem-dependent movement, SMV moves from the mesophyll via bundle sheath cells, phloem parenchyma, and companion cells into phloem sieve elements (SEs), and are then passively transported following the source-to-sink flow of photo assimilates and unloaded from SEs to sink tissues (distant sites) from which further infection will occur. CP, HC-Pro, VPg and 6K2 have been identified as key players in systemic infection (Cronin *et al*., 1995; Dufresne *et al*., 2008; Thivierge *et al*., 2008; Wei *et al*., 2010b).

1.2.8 Transmission of SMV

SMV is a seed-borne and seed-transmitted virus (Domier *et al*., 2007). Up to 30% or more of the seeds from infected soybean plants carry SMV depending on cultivar and time of infection (Bos, 1972).

Apart from seed transmission, SMV is transmitted also by mechanical inoculation and aphid vectors. Over 30 aphid species can transmit the virus in a non-persistent manner (Maury, 1985). Transmission via aphids is dependent on SMV strains and aphid species. Although some strains of SMV are hardly aphid transmissible (Takahashi *et al*., 1980), SMV strain G5 (a non-transmissible isolate) recovers its aphid-transmission property when plants are also co-infected with an aphid-transmissible SMV strain (Cho, 1981).

The non-transmissible SMV-G5 strain probably lacks the helper-component, which is essential for aphid transmission. When plants are infected with both transmissible and non-transmissible viruses, the non-transmissible virus takes the advantage of the helper component of the transmissible virus enabling them for aphid transmission (Matthews, 1991).

1.2.9 Seed transmission of SMV

SMV-infected seeds are the primary inoculum source, though weeds and other plants may also serve as a reservoir of SMV. Thus, SMV transmission through seeds plays an important role in the epidemiology of the virus (Maury, 1985).

When a soybean has reached vegetative maturity (12 to 14 inches tall, 6 nodes), it will enter the reproductive phase, which can be artificially divided into 8 stages, i.e., R1-beginning bloom, R2-full bloom, R3-beginning pod, R4- full pod, R5- beginning seed, R6- full seed, R7-semi mature seed and R8- full mature seed. A mature soybean seed consists of the seed coat and the embryo. The embryo is composed of the radicle and cotyledons (Goldberg *et al*., 1994; Moise *et al*., 2005; Singh, 2010).

Seed transmission of SMV is dependent on the ability of the virus to survive in the embryo during seed maturation (Bowers and Goodman, 1991; Bowers Jr and Goodman, 1979). Seed transmission is successful when the virus has the ability to move into and replicate within reproductive tissues (Johansen *et al*., 1994). Seed transmission rates are dependent on the cultivar, the isolate and the time of infection before flowering (Bowers

and Goodman, 1991). Viruses can infect the embryo either directly by invading the embryo (Maule and Wang, 1996) or indirectly by infecting the megaspore or the pollen mother cells before embryo formation. A previous study has shown that another potyvirus member, *Pea seed-borne mosaic virus* (PSbMV) infects embryos directly through symplastic connections between the maternal cells and the embryo (Roberts *et al.*, 2003). From the maternal tissue, PSbMV enters into the endosperm through plasmodesmal channels between the endosperm cells and testa. During the early seed development stage, PSbMV invades the suspensor cells through transient vesicles which are 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. However, at the early seed development stage, the presence of the virus in the micropylar region is necessary for seed transmission (Roberts *et al*., 2003).

As mentioned above, SMV is a seed-transmitted virus that has been shown to cause severe consequences on plant growth and seed quality (Zheng *et al.*, 2005). Blocking seed transmission could be a novel method to control SMV infection. Unfortunately, the molecular mechanisms behind SMV seed transmission remain obscure and little is known about how the virus moves from sporophytic to gametophytic tissues and back into sporophytic tissue or from the infected maternal tester to the embryo. Also, it is unclear which molecular functions SMV undergoes in infected embryos, how it survives and when and where SMV starts replication during seed germination and seedling growth stage. My proposed study deals with the characterization of seed transmission of SMV in soybean.

1.3 Hypothesis and Objectives

Even though a lot of information is available regarding the seed transmission of plant viruses, most of it is at the diagnostic level. By comparison, relatively little research has been done to elucidate the molecular mechanisms of seed transmission. The goal of my research was to understand the activities of SMV in soybean seeds. Knowledge generated in this study would assist in the developing of novel antiviral strategies to block virus seed transmission. I hypothesized that SMV transmission to seeds proceeds via a route from the infected maternal tester to the embryo. I also hypothesized that SMV starts replication in the embryo before dormancy and pauses during dormancy (storage) but reactivates after seed germination. Lastly, I hypothesized that SMV replication continues during the seedling growth stages. The specific objectives of this research were:

1) To determine the efficiency of SMV transmission in soybean seeds.

2) To determine if and at what level SMV replicates in SMV-infected embryos.

3) To understand when and where SMV starts replicating during seed germination and early seedling stages.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

In planta SMV experiments were performed at the Southern Crop Protection and Food Research Center (SCPFRC) in London, Ontario. Soybean plants were planted in an aphid free growth chamber with 16 h of light at 22 ℃, 8 h of darkness at 18 ℃ and 75% to 80% relative humidity (RH) conditions. Soybean cultivars Williams 82 (susceptible host, no resistance gene), PI 96983 (carrying resistance gene *Rsv1*) and V94-5152 (*Rsv4*) were used in this study.

2.2 Mechanical Inoculation

A green fluorescent protein (GFP)-tagged recombinant SMV infectious clone derived from an SMV London isolate (Figure 3) was introduced into Williams 82 seedlings via biolistic bombardment (Yamagishi *et al*., 2006). Systemically infected leaves were subsequently used as an inoculum to infect two week old soybean plants by mechanical inoculation. Briefly, approximately 1 g of infected leaf tissue was harvested and homogenized with a mortar and pestle in 5 mL of 0.01 M potassium phosphate buffer, pH 8.0 (Sambrook and Russell, 2001). The inoculum was applied to both unifoliate and 1st trifoliate leaves of soybean seedlings predusted with carborundum (Zheng *et al*., 2005).

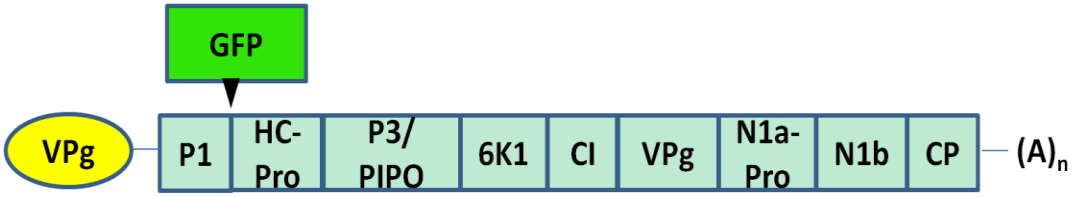

Figure 3: Location of the GFP insert between P1 and HC-Pro of SMV cDNA. An infectious SMV-L-GFP clone was created, where a green fluorescent protein (GFP) gene was inserted between P1 and HC-Pro.

2.3 Virus detection in vegetative tissues of soybean plants

2.3.1 Detection by ELISA

Plants were observed for symptom development following virus inoculation. The presence of SMV was detected using a commercial double-antibody sandwich ELISA (DAS-ELISA) kit (Agdia, Inc., SRA 33300) following the manufacturers protocol. Absorbance was measured at 405 nm using an iMARK^{TM} absorbance micro plate reader (Bio-Rad Laboratories Inc).

2.3.2 Detection by RT-PCR

2.3.2.1 Total RNA extraction:

Virus-infected and control leaf tissues (50-100 mg) were collected into 2 mL vials, containing 3 plastic beads (2.3 mm diameter, BioSpec Products) for homogenization, and immediately placed into liquid nitrogen for 2 to 3 min. Once tissues were thoroughly frozen, vials were placed into Tissue lyser adapter plates (Qiagen) for homogenization. Tissue samples were homogenized using the TissueLyser for 45s (30 Hz). Following homogenization, 1 mL of Trizol® reagent (Qiagen) was added to each sample. After vortexing for 15 s, samples were incubated at room temperature for 5 min to permit dissociation of nucleoprotein complexes. Next, 200 µL of chloroform was added and samples shaken by hand for 15 s. Samples were then centrifuged (12000 x *g*) for 15 min at 4 ℃. Following centrifugation the aqueous phase was transferred to a 1.5 mL tube containing 500 µL of isopropyl alcohol for precipitation. Following a 15 min incubation

on ice, the RNA was pelleted by centrifuging for 10 min at 4 ℃. The RNA pellet was washed in 1mL of 70% ethanol, and centrifuged for five minutes at 7500 x *g*. The pellet was then air-dried and re-suspended in 50 µL of nucleotide free H_2O and incubated in a water bath or a heated block set at 55-60 ℃ for 10-15 min. The quality and concentration of RNA was determined using a nanodrop spectrophotometer (Thermo Fisher Scientific, USA) and by gel electrophoresis on a 1% agarose gel.

2.3.2.2 Preparation of RNA samples prior to RT-PCR:

To remove all genomic DNA from the RNA preparation, the RNA samples were treated with DNase I (Catalog no. 18068-015, Life technologies) before proceeding to all downstream applications. Briefly, an RNase-free micro centrifuge tube containing 1 µg RNA sample was added with 1 µL of DNase I (Amplification Grade, 1 U/µL) in a 10 µL reaction. Following a 15 min incubation at room temperature, 1 µL of 25 mM EDTA solution was added to the reaction mixture to inactivate the DNase I. The reaction mixture was then heated for 10 min at 65 ℃. The RNA sample was then ready to use for a reverse transcription reaction. Purified RNA from both infected and mock inoculated leaf tissue was used for RT-PCR, using SMV specific primers to detect the presence of SMV.

2.3.2.3 cDNA preparation and RT-PCR

A Superscript II first strand synthesis kit (Cat. No. 18064-022, Life technologies, Canada) was used to synthesize cDNA from 1 µg of RNA and oligonucleotide dT20. Each reaction was set up according to the manufacturer's protocol in the presence of 1 µL of RNase inhibitor (Invitrogen). CP gene specific primers, CP-F and CP-R (Table 1) were designed for the detection of SMV by RT-PCR. Amplification of elongation factor alpha (EF-α) and 18s rRNA was used as a positive control. Amplifications were performed in a thermal cycler with each 50 µL reaction containing 10X PCR Buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 50 mM $MgCl_2$, 10 mM dNTP mix, Taq DNA polymerase (5 U/µL), forward and reverse primer (10 µM) (Table 1) and 100 ng of cDNA template. Cycling conditions were 94 ℃ for 3 min of initial denaturation, followed by 40 cycles of 94 ℃ for 20 s, annealing at 50-60 ℃ for 30 s to 60 s (depending on primer annealing temperature and size of target band) and 72 ℃ for 1 min. This was followed by a 72 ℃ final extension for 3 min.

2.3.3 Detection by Confocal microscopy

Soybean tissue infected by SMV-GFP was imaged at room temperature using a Leica TCS SP2 inverted confocal microscope with an argon ion laser. GFP was excited at 488 nm and the emitted light was captured at 505 to 555 nm. Light emitted at 630–680 nm was used to record chlorophyll autofluorescence. Images were captured digitally and processed using the Leica LCS software.

2.4 Seed collection, treatment and sterilization

Different reproductive stages of seed from infected and mock infected Williams 82 soybean plants were monitored. Reproductive tissues such as floral tissues (R1 to R2) and seed pod (R4 to R8) were collected during different developmental time phases. Once collected, immediately stored at -80 ºC or processed right away for RNA isolation and downstream processing using molecular biology techniques. For confocal, immuno-fluorescence and electron microscopy, young leaf tissues and different seed stages were immediately processed following collection. Ten seed pods from the same reproductive stage of different infected plants were randomly collected along with 2-3 seed pods from uninfected soybean plants to be used as a negative control.

Mature seed pods were collected from infected and healthy plants. Mottled and non-mottled seeds were separated manually and stored at room temperature. All mature seeds were surface sterilized for seed germination experiments.

For seed sterilization, mature soybean seeds were placed in 100 x15 mm Petri dishes. Petri dishes were placed in a bell jar desiccator along with a 250 mL beaker containing bleach (sodium hypochlorite) and concentrated (12 N) HCl, whose combination releases chlorine gas. Following an overnight (16 h) exposure to chlorine gas, Petri dishes were left closed and removed them from the bell jar and placed in a laminar flow hood. Within the hood, plates were opened and allowed to air out for 30 min to remove the excessive chlorine gas.

2.5 Visualization of green fluorescence of seed tissues at different reproductive stages of seeds.

2.5.1 Visual detection of green fluorescence in the seed tissue

To understand and detect the infection of SMV within seed embryos, early reproductive stages of seeds were collected and prepared for green fluorescence visualization using confocal microscopy. Floral parts and immature seeds (from stages, R1-R4) were randomly collected for Williams 82 soybean plants infected by SMV-L-GFP, sectioned using a scalpel and then immediately slide mounted for the visualization of green fluorescence. Seeds at R6-R8 reproductive stages were dissected into the seed coat, cotyledon and radical. Seed coat tissue was obtained in small sections from around the micropyle. The radical was serially sectioned so that the laser could pass through the tissue. To visualize SMV-L-GFP in seed tissue, a Leica DM IRE2 confocal microscope was used.

After sterilization, mature infected seeds were germinated in MS medium (Appendix B) for 3 to 5 days until the cotyledon stage was reached. Shoots and cotyledon leaves were separated and immediately dissected for GFP visualization. GFP visualization was done using the above mentioned protocol with confocal microscopy.

2.5.2 ELISA detection of SMV in seed tissues

Seeds at the R8 stages were dissected into the cotyledon, radicle and seed coat under sterile conditions. The cotyledons were ground into a mortar and pestle in presence of

liquid nitrogen followed by protein extraction into a 300 µL of GEB buffer (Agdia, Inc., SRA 33300). The radicle and seed coat tissue were ground using Tissue Lyser II (Qiagen) at a frequency of 30Hz for 60 s with copper head beads. Protein was extracted from samples by adding 300 µL of GEB buffer (Agdia, Inc., SRA 33300). Confirmation of infection was determined using an SMV DAS-ELISA kit (Agdia-SRA 33300) following the manufacturer's protocol.

2.5.3 RT-PCR detection of SMV in seed tissues

Seeds at the R4, R6, R7 and R8 stages were dissected into the cotyledon, radicle and seed coat under sterile conditions. RNA from reproductive tissues of seeds was extracted using Trizol® reagent (Qiagen) and cDNA was prepared using the Super Script II First Strand Synthesis kit. To confirm the presence of SMV in the seed coat and embryo, a 794 bp cDNA region of the CP gene was amplified using primers CP-F and CP-R (Table 1). For the positive control, the house keeping gene elongation factor was amplified using primers, EF-α-F and EF-α-R (Table 1). The PCR reaction was run for 40 cycles with parameters described in 2.3.2.3.

Table 1. List of primers used in this study

Primer Name	**Primer Sequence (5′ to 3′)**	**Target gene**
CP -F	TCAGGCAAGGAGAAGGAAGG	CP
CP -R	CTGCGGTGGGCCCATGC	CP
CP –R2	CTTCTGCAAACGCGGAACCA	CP
EF-α-F	GATGCCACTACCCCGAAGT	EF-α
EF-α-R	AGACATCCTFCAATGGAAGC	EF-α
18S rRNA-F	GGGCATTCGTATTTCATAGTCAGAG	18S rRNA
18S rRNA-R	CGGTTCTTGATTAATGAAAACATCCT	18S rRNA

2.6 Evaluation of SMV accumulation in the seed embryo by quantitative RT-PCR

SMV accumulation in seeds at different reproductive stages was analyzed by quantitative RT-PCR (qRT-PCR) using CFX96 Touch™ Real-Time PCR Detection System (BioRad). Prior to conducting qPCR, the PCR assay was tested to ensure optimal annealing temperature, efficiency and specificity of the reaction. To achieve reliable data through qRT-PCR, the guidelines for the minimum information for publication of quantitative real-time PCR experiments (MIQE) were also followed (Bustin *et al.*, 2009; Taylor, 2010).

Following RNA extraction, the RNA was treated with DNAse I (Ambion, USA) to eliminate any genomic DNA contamination in the sample. The DNAase was then removed using DNAase inactivation reagents (Ambion, USA) as per the manufacturer's instructions. The reverse transcription reaction was performed as per the above mentioned protocol.

To determine the efficiency of the assay, serial dilutions of the pooled cDNA was used in the reaction to generate a standard curve. The cDNA was diluted with sterilized distilled water (1:3) and 2 µL of the diluted cDNA was used in the PCR reaction. Relative amounts of CP mRNAs were calculated from threshold cycle values. The *18s rRNA* reference gene was used for normalization. Primers, CP-F and CP-R2 (Table 1) were used for detection of CP gene expression at different embryonic stages of seed. All results were shown as mean of at least three biological replicates with corresponding standard errors.

A total volume of 20 µL containing 0.5 µM of each forward and reverse primer, 2X SensiFast® Syber No-Rox mix (Bioloine), 2 µL cDNA was used in each qRT-PCR reaction. For each experiment, at least three replicates were tested. The PCR was performed in two steps; 95 ℃ for 3 min followed by 45 cycles at 95 ℃ for 10 s and 60 ℃ for 30 s. Finally, the transcript levels of the target genes were analyzed using relative quantification by comparative Ct (2-ΔΔCT) method (Livak and Schmittgen, 2001).

2.7 Detection of SMV-induced cytological abnormalities by TEM

2.7.1 Tissue fixation, embedding, and sectioning by ultra microtome

Systemically infected leaf tissues and seeds were harvested and cut into 1-2 mm^3 pieces and were then placed into a fixative solution (2% glutaraldehyde and 4% paraformaldehyde buffered to pH 7.2 with 0.1M potassium phosphate buffer) and left overnight at 4 ℃ for fixation. Vacuum infiltration was performed in a bell jar during the fixation process. After washing twice with phosphate buffer (20 minutes each time), tissues were added with 50 mM ammonium chloride (prepared in 1M potassium phosphate buffer, pH 7.2) to remove aldehydes. After 3X rinse in double distilled water for 5 minute each time, the tissues were dehydrated with a graded series of ethanol and embedded in London Resin White (Cederlane, Canada). Polymerization was carried out in embedding molds at 60 ℃ for 24 h and cooled for another 24 h. Excess plastic surrounding the tissue was trimmed using a razor blade in a fashion that yielded a square or rectangular tissue section. This plastic capsule was trimmed to a pyramid shape.

Ultrathin (60-70 nm) sections were cut with a diamond knife on an ultramicrotome and were mounted on 400 mesh nickel grids. Nickel grids were then stained with 2% uranyl acetate and 2% lead citrate for 5 min each. Electron microscopy was carried out in a Philips CM10 transmission electron microscope at the Biotron or TEM JEM 1011 (JEOL Ltd., Tokyo, Japan) at Agriculture and Agrifood Canada.

2.8 Detection of SMV by immunogold labeling experiment

2.8.1 Tissue fixation, embedding, and sectioning by ultra microtome

For immunogold labeling, leaf tissue and seed samples were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2). Tissues were dehydrated by increasing ethanol series, and embedded in LR White resin. Polymerization was carried out for 24 h at 60 ℃. Ultrathin serial sections were cut with an ultra-microtome and mounted on formvar-coated 400 mesh nickel grids.

2.8.2 Immunolabeling

Immunogold labeling was carried out by floating the grids carrying serial tissue sections for 30 min at room temperature in phosphate buffer saline buffer (pH 7.4). After incubation for 30 minutes at RT in Aurion blocking solution (20 mM PBS, 0.1-0.2% and 15 mM NaN_3, pH 7.4), the grids were incubated for 20 min at 37 °C with primary antibody diluted to 1/500 in blocking solution. The grids were washed twice for 30 min each with PBS-Tween 20 and incubated for 45 min with secondary antibody conjugated

to 25 nm colloidal gold particles that had been diluted in blocking solution. After extensive washing with PBS and ultrapure water, sections were stained with 2% uranyl acetate and 2% lead citrate. After staining, grids were washed twice with ultrapure water and kept on Whatman filter paper on a Petri dish for drying overnight. On the following day, electron microscopy was carried out in a Philips CM10 electron microscope or a TEM JEM 1011 microscope (JEOL Ltd., Tokyo, Japan).

As a negative control, leaf and seeds from healthy Williams 82 plants were used and processed as per the above mentioned protocol.

2.9 Detection of negative sense RNA by *in situ* hybridization

In situ hybridization was performed using the protocol of Karlgren et al. (2009), Simon's lab (open access protocol online, see references), Tadege and Kramer labs (open access protocol online, see references) and Karen Nygard (Personal communication at Biotron) with some modifications and changes to adapt to our tissue type. The modified protocol consists of steps including tissue fixation and embedding, washes, pre-hybridization, hybridization, blocking, antibody incubation and finally treatment with a color reagent.

2.9.1 Tissue collection, fixation, embedding, and sectioning

Infected leaf tissue samples and immature seed tissues at R3-R4 stages were prefixed at FAA (37% Formaldehyde/Glacial Acetic Acid/Alcohol) solutions overnight. After

fixation, samples were sent to Robarts Molecular Pathology facility at Western University for automated tissue processing and wax embedding. Thick sections (10 µm) were cut using a Leitz wax microtome at the Biotron Integrated Microscopy Facility and collected onto Superfrost® plus pre-cleaned microscope slides (Fisher Scientific, Toronto Canada). Sections were placed onto the slide by floating on RNase-free water in the wells created by the paraffin crayon, then removal of the water by wicking with a kimwipe. After drying down at 42 ℃ overnight, slides were checked by visual inspection using a dissecting microscope. Serial sections were deparaffinized by three incubations of 10 min each in Histochoice clearing solution (Sigma Aldrich, St. Louis, MO, USA) before rehydration in an ethanol gradient series (100%, 90% and 70%) up to DEPEC- treated water. Slides were then subjected to a Proteinase-K stock solution (final concentration, 0.2 U/mL; 37 ℃ for 30 min) to partially digest cross-linked proteins from fixation, and allow the probe to access its target. Serial sections were then dehydrated through graded RNAse free ethanol series (through a 100, 100, 95, 85, 70, 50, and 30% ethanol series for 30 seconds each) , then either used immediately, or air dried and stored at -80 °C with desiccant, if not hybridized on same day.

2.9.2 Probe preparation

SMV-CP gene nucleotide sequences were targeted for RNA probe preparation to investigate negative sense RNA replication in SMV. First, SMV gene-specific forward and reverse primers were used for amplification of sense DNA templates from the SMV specific cDNA, using the PCR DIG Probe Synthesis Kit (Roche, Germany). To all

fragments, a T7 overhang was then added using primers carrying the T7 sequence. All primers were designed with GC content near 50% and melting temperatures near 60 °C. PCR products were subsequently purified using the high pure PCR Product Purification Kit (Roche, Germany) according to the kit manual, and the PCR products were eluted with 50 µL DEPEC water.

Generation of DIG labeled sense probes from these cDNA templates were performed by *in vitro* transcription with T7 RNA polymerase using the DIG RNA Labelling Kit (SP7/T7) from Roche (Roche, Germany) according to the kit manual. DIG labeled probes were purified by adding 4 µL 4 M $LiCl_2$ and 50 µL of 100% ethanol and incubating at -80 °C for 1 h. Centrifugation was done at the maximum speed (approximately 16,000 x g) for 10 min in a bench top microfuge to pellet the probe. This centrifugation enabled the removal of the ethanol mixture before washing probes with 70% ethanol. Probes were centrifuged for another 10 min followed by the removal of ethanol and were then allowed to air dry and were finally suspended in 50 µL of RNase-free DEPC water and stored at -80 °C.

2.9.3 Probe efficiency measurement

DIG labeled RNA probes were quantified using a spot test according to the DIG RNA Labelling (SP7/T7) kit manual. A Spot test was done in positively charged Hybond N+ nylon membranes (Amersham Biosciences LTD, United Kingdom). Colorimetric detection was performed using Roche wash and block buffer Set (Roche, Germany), anti-DIG AP, Fab fragments (Roche, Germany) and color development with NBT/BCIP

(Roche, Germany). Briefly, after cross-linking with UV cross linker (1200 µJ/cm^2), the spotted membrane was washed once in wash buffer for 5 minutes followed by a 60 min incubation with blocking buffer, followed by an incubation with anti-DIG AP solution (75 mU/mL in 1X maleic acid buffer [0.1 M, pH 7.5]). Then, the membrane was rinsed and washed with wash buffer twice for 15 min each and was incubated in coloring reaction solution (18.75 mg/mL NBT, 9.4 mg/mL BCIP) in the dark until detection of probe spots was sufficient (up to 24 h with periodic checking). All detection steps were carried out under constant agitation.

2.9.4 Hybridization to tissue samples and color development

Once the sense probe was ready for hybridization, slides containing tissue samples were placed in pre-hybridization buffer solution (without probe) (Appendix C) at 55 ℃ for one hour. Lastly, slides were placed in hybridization buffer with probe concentration of 0.5 µg/mL at 55 ℃ for overnight static incubation in a hybridization chamber.

On the following day, the slides were removed from hybridization buffer and washed in 2X SSC and 1X SSC each twice for 10 min at 37 °C, followed by two washes with 0.1X SSC at 37 °C for 30 min each, to reduce nonspecific binding reactions. All SSC washes were conducted on a shaker at 37 °C.

Then, the tissue was statically incubated in Boehringer blocking solution (Appendix C) for 1 h at room temperature, followed by 2 h of incubation on a shaker at 37 ℃ with anti-digoxygenin-alkalaine phosphatase (AP) antibody (Roche, Germany). Subsequently, the

tissue was washed for 10 min each in Buffer 2 (pH 7.5) and Buffer 3 (pH 9.5) (Appendix C) at room temperature on a shaker. The increase in pH from 7.5 of Buffer 2 to 9.5 of Buffer 3 prepares the tissues for final incubation in BCIP/NBT alkaline phosphatase substrate solution for color development. Experimental samples and negative controls were placed in this BCIP/NBT solution, and the reaction was monitored carefully for colour development. Then, both control and experimental tissue reactions were stopped simultaneously for consistency, by adding stop solution or Buffer 3 (Appendix C). As BCIP/NBT solutions were light sensitive, this staining protocol was performed in dark condition. Images were taken at 4X and 10X using a Nikon digital camera (Nikon, DXM1200) by ACT-1 software (Nikon, version 2.1.2).

2.10 Detection of double stranded RNA (dsRNA) by immunofluorescence

Immunofluorescence staining was performed using the protocol of Karen Nygard (Personal Communication) with some changes to adapt to our tissue type.

2.10.1 Tissue collection, fixation, embedding, and sectioning by Wax microtome

To detect SMV replication in seed embryo, soybean plant leaf and immature early stages of seeds (R3 to R5) were randomly picked and immediately fixed in FAA (50% ethanol, 5% Acetic acid, and 5% formaldehyde), and sent to Robarts Molecular Pathology facility at Western University for tissue embedding in Paraplast-Xtra (Fisher Scientific). The embedded samples were sectioned into 10 µm thick slices using wax microtome (Leitz) in the Integrated Microscopy Facility at Biotron, Western University and sections were

carefully collected onto Super Frost Plus microscope slides (Fisher Scientific, Ottawa, Canada). After drying at 42 °C overnight, slides were checked by visual inspection using a light microscope. Slides were then stored at -80 °C until use.

Serial sections were deparaffinized by three incubations of 5 min each in xylene before rehydration in an ethanol gradient series (100%, 90% and 70% Ethanol) up to diethylpyrocarbonate-treated (DEPC) water. Subsequently, the tissues were rinsed in reverse osmosis water for one minute and in Phosphate buffered saline (PBS) 3X for 5 minute each. At the next step, the tissues were incubated with 1% Triton X-100 at room temperature for 1 h followed by rinsing with PBS thrice for 5 min each.

2.10.2 Blocking, incubation and mounting

For immunofluorescence studies, all incubations were carried out in a covered, light proof humidity chamber at room temperature. Tissue samples were blocked in Background Sniper (Biocare Medical, Concord CA USA) for 10 min and then rinsed briefly, with PBS. Subsequently, the tissues were incubated with dsRNA-specific primary antibody J2 (Scicons, Hungary), at 1:500 dilution in Dako Universal Antibody Solution (Dako Canada Inc, Burlington, ON) for 1 h. After incubation, the excess antibody was rinsed off with 3X for 5 minutes each with PBS, to remove traces of unbound J2 antibody. Samples were then incubated in Alexa Fluor 594-conjugated anti-mouse IgG secondary antibody (Molecular Probes/Life Techologies Inc, Burlington ON) at 1:200 dilutions for 1 h. Excess secondary antibody was rinsed off and washed twice with PBS for 5 min each. Finally, the samples were mounted using aqueous anti-fading mountant (Dako, Canada). Mounted slides were then kept for 24 h in flat position at room

temperature in the dark, and then the slide edges were sealed with nail polish and stored at 4 ℃ until analyzed under a fluorescent microscope.

2.10.3 Image analysis

After immunofluorescence staining, the sections were imaged using a Zeiss AxioImager Z1 fluorescence microscope (Carl Zeiss Ltd. Oberkocken, Germany). DAPI images were captured using a dichroic filter with excitation BP 390/22 nm and emission BP 460/50 nm. DsRed images were captured using a filter with excitation BP565/30nm and emission BP620/60nm. Colocalization of two markers was verified by merging the images from the two channels. Images were captured digitally and were processed using the Zeiss Zen software (Carl Zeiss Ltd. Oberkocken, Germany).

2.11 Seed transmission

Seed transmission efficiency of SMV in Williams 82 were obtained by harvesting mature seeds from SMV infected plants that were grown in greenhouse conditions. A total of 15 infected mature plants were selected in 2013-2015 to harvest the mature seed pods. A total 120 mottled mature seeds and 90 non-mottled mature seeds were planted for detection of SMV transmission. Once seeds had germinated and reached the first trifoliate leaf stage after 2-3 weeks, SMV infection in the young plants was detected for typical symptoms in the leaf. ELISA and confocal microscopy were also used to detect the presence of SMV.

3 RESULTS

3.1 Symptom development

The susceptible cultivar Williams 82, which does not have any resistance genes, and two resistant accessions PI96983 carrying the resistance gene *Rsv*1 and PI596752 with *Rsv*4 were used in this study. All the plants were grown in an aphid free growth chamber. Systemically infected soybean leaves from Williams 82 seedlings bombarded with SMV-L-GFP were used as an inoculum to mechanically inoculate 3-week-old Williams 82 soybean plants and the two resistant varieties listed above. The Williams 82 cultivar showed symptoms in leaves, whereas, no symptoms were evident in the resistant cultivars (PI 96983 and PI 596752). The earliest symptoms in Williams 82 appeared as a mild mosaic pattern in the first trifoliate leaves 7 days post inoculation (dpi) and gradually became more severe at 14 dpi (Figure 4a). Leaf symptoms further developed into severe mosaic with mild mottling at about 21-28 dpi (Figure 4b and 4c). Some of the Williams 82 plants at this stage also showed yellowing in the leaf veins, leaf chlorosis (Figure 4d) and wrinkling. Plants carrying the either *Rsv*1 or *Rsv*4 resistance gene showed no symptom development after inoculation with SMV-L-GFP (Figure 5), confirming that SMV-L-GFP is unable to infect these resistant cultivars. The infected Williams 82 plants were used to study SMV seed transmission.

3.2 ELISA and RT-PCR detection of SMV from infected plant tissue

The accumulation of viral CP from SMV-infected Williams 82 leaf tissue was detected by ELISA. The clearly visible yellow color in the wells of the ELISA plate confirmed the

(a) Mosaic symptom in the leaf

b) Mottling and Mosaic together in the leaf

c) Leaf mottling

d) Leaf chlorosis

Figure 4: Symptoms in Williams 82 soybean plants inoculated with SMV-L-GFP. (a), Soybean plants (Williams 82) inoculated with SMV-L-GFP showed severe mosaic pattern in the first trifoliate leaves at 14 dpi. (b) and (c), Leaf symptoms further developed into severe mosaic with mild mottling at about 21-28 dpi. (d) Some of the SMV infected Williams 82 plants also showed chlorosis in the leaves.

Figure 5: Leaves from Williams 82 inoculated with buffer and PI96983 (*Rsv*1)/ PI596752 (*Rsv*4) inoculated with SMV-L-GFP. Soybean plants (Williams 82) inoculated with virus inoculation buffer did not exhibit any virus symptom in the plant (Upper panel). Similarly, no virus/symptom evident when the *Rsv*1/ *Rsv*4 plant inoculated with SMV-L-GFP (Bottom panel).

presence of the viral CP in the samples (Figure 6). However, as expected, no virus was detected in the samples from mock-inoculated Williams 82 plants or virus inoculated PI96983 and PI596752 leaf tissues, as the wells containing these samples remained colorless (Figure 6). Absorbance was measured at 405 nm on an iMARK^{TM} absorbance micro plate reader (Bio-Rad Laboratories Inc.). The optical density (OD) values of SMV-infected leaf tissue samples were between 0.75-1.00, which is very similar to the positive control OD absorbance values of 0.70 to 1.00, and were significantly different than the negative control or mock OD values (Table 2).

Using RT-PCR with SMV CP-specific primers, the presence of SMV in the systemically infected leaves was confirmed. A 794 bp RT-PCR product of SMV CP was detected in all Williams 82 plants inoculated with SMV-L-GFP that also exhibited symptom development (Figure 7). However, no specific SMV CP RT-PCR product was detectable in the resistant plants that were infected with SMV-L-GFP.

3.3 Monitoring of SMV infection in soybean leaf tissues by confocal microscopy

Systemic infection by SMV-L-GFP in virally infected Williams 82 was monitored by confocal microscopy. Leaf tissue samples were collected from infected plants or mock inoculated Williams 82 soybeans and resistant (*Rsv1*) plants. Chloroplast auto-fluorescence was monitored and recorded at 680 nm. GFP was excited at 488 nm and the emitted light was captured at 505 to 525 nm. GFP fluorescence in epidermal cells of leaves distant from the inoculated leaf was taken as evidence of systemic infection by

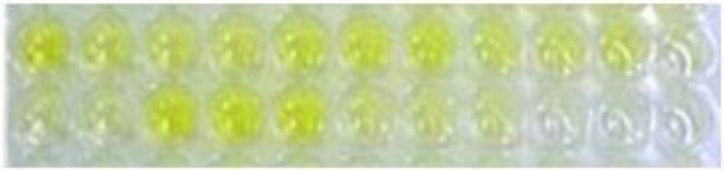

Figure 6: ELISA assay. Viral coat protein in the sample was recognized by SMV-specific antibodies in microtiter plates. A yellow coloration confirmed viral infection whereas uninfected samples remained colorless. N denotes negative controls. N1, mock-inoculated Williams 82 plant; N2, *Rsv 1* plant (PI96983) inoculated with SMV-L-GFP; N3, *Rsv 4* plant (PI596752) inoculated with SMV-L-GFP.

Table 2. A statistical analysis of ELISA data for SMV detection.

Cultivars	**Infected leaf (Data Range)**	**Healthy leaf (Data Range)**	**Positive control (Data Range)**
Williams 82	0.955 A * (0.75-1.000)	0.161 A (0.12 -0. 21)	0.92 A * (0.70-1.000)
PI96983 (*Rsv1*)	0.12 A (0.114-0.129)	0.15 A (0.12-0.21)	
PI596752 (*Rsv*4)	0.117 A (0.10-0.12)	0.12 A (0.12-0.14)	

Note: * The mean value of ELISA absorbance of the sample from infected Williams 82 plants is statistically significantly higher than that of the mock-inoculated plant (negative control) ($P < 0.05$), whereas the mean values from *Rsv*1 and *Rsv*4 samples show no significant difference from that of the mock-inoculated plant ($P > 0.05$). Student t-test was used in this study to determine the significance of the result.

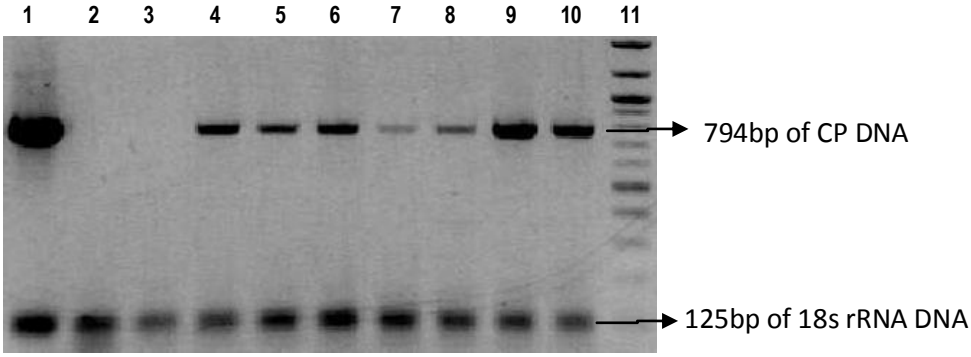

Figure 7: Agarose gel electrophoresis analysis of RT-PCR products. A 794 bp RT-PCR product resulting from amplification of the SMV CP coding sequence was detected. Amplification of 125 bp of 18S rRNA was used as a control. Lane 1 indicates positive control and Lane 4 to 10 shows amplification of *CP* and 18s *rRNA*, respectively, from different SMV infected plant tissue. No *CP* amplicon was observed from *Rsv1* and *Rsv4* plant samples in lanes 2 and 3. Lane 11 indicates different size of the 100 bp ladder.

SMV (Figure 8). No green fluorescence was detected in the leaf sample from the healthy control plant (Figure 8).

3.4 Monitoring of SMV infection in soybean floral tissues at R1/R2 stages by confocal microscopy.

To investigate the pattern of SMV-L-GFP distribution in floral organs during plant development, eight different regions of plant floral tissue were collected. Petal and sepal tissue were separated from each flower. GFP accumulation was detected in the epidermal cells of the flower petals (Figure 9, upper panels) and sepal (Figure 9, middle panels) at the R2 stage, indicating the presence of SMV in the flower. As expected, no green fluorescence signal was detected in the floral tissue of mock inoculated plants (Figure 9, bottom panels).

3.5 ELISA and RT-PCR detection of SMV from floral (R1/R2 Stage) tissues

ELISA and RT-PCR were further used to detect SMV in soybean floral tissues. Initial ELISA analysis revealed that no distinct positive signals were detected in some floral tissue samples from SMV-L-GFP infected soybean plants while some other floral tissue from infected soybean plants showed SMV-positive results (data not shown). It is well known that as a protein-based detection technology for virus detection, ELISA can lead to false negative diagnosis results with low virus concentrations in the floral tissue

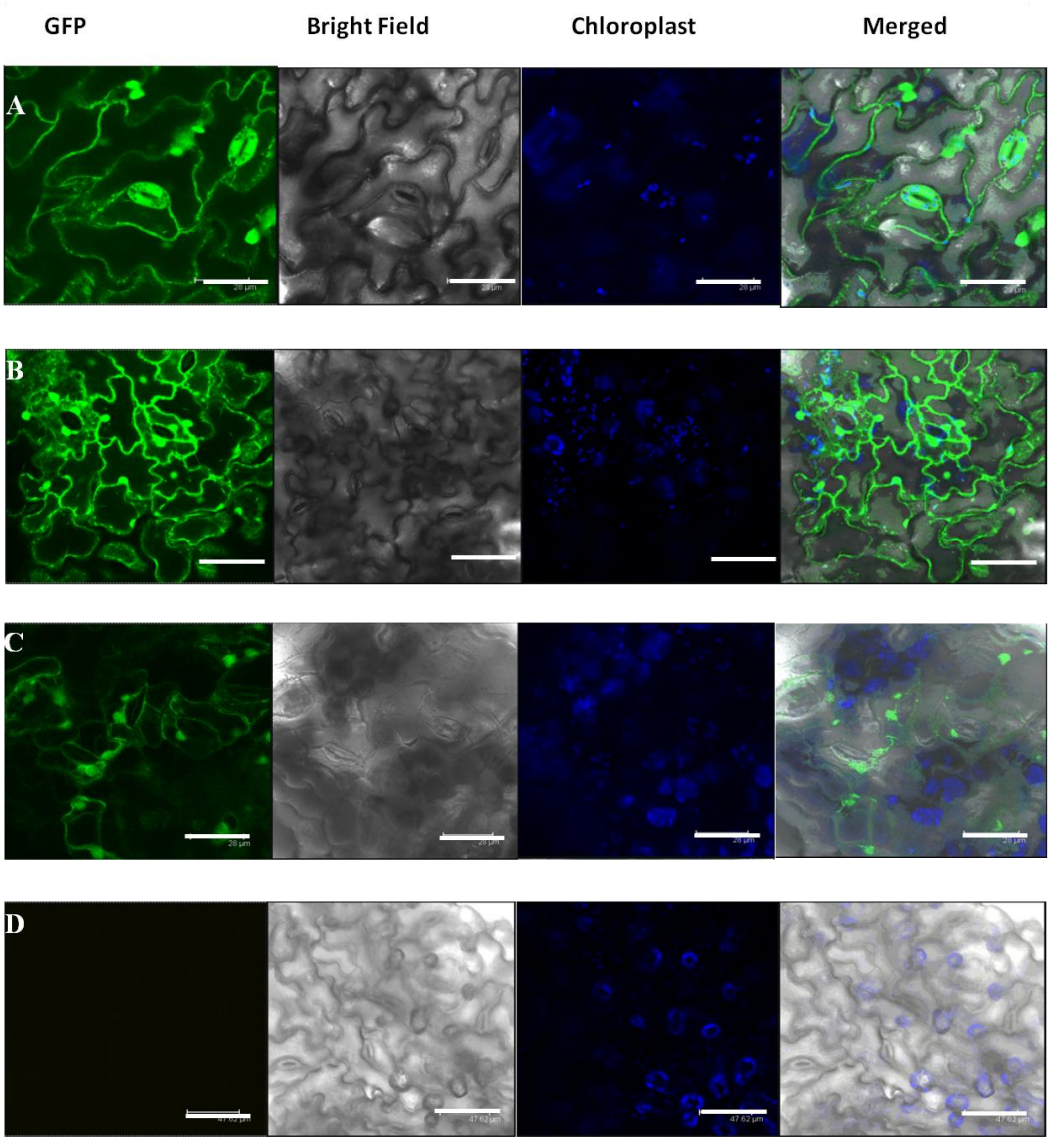

Figure 8: Confocal microscopy of infected leaf tissues. Infected leaf tissue samples showed GFP fluorescence in the epidermal cells. Chloroplast auto fluorescence is indicated by blue color. Uninfected tissue samples showed no GFP fluorescence under the same conditions. Upper panels A to C are images from leaf samples collected from three different infected plants, and bottom panel D is from a healthy plant (Mock - Williams 82). Bars in (A and C): 28 micron and in (B and D): 47.62 micron.

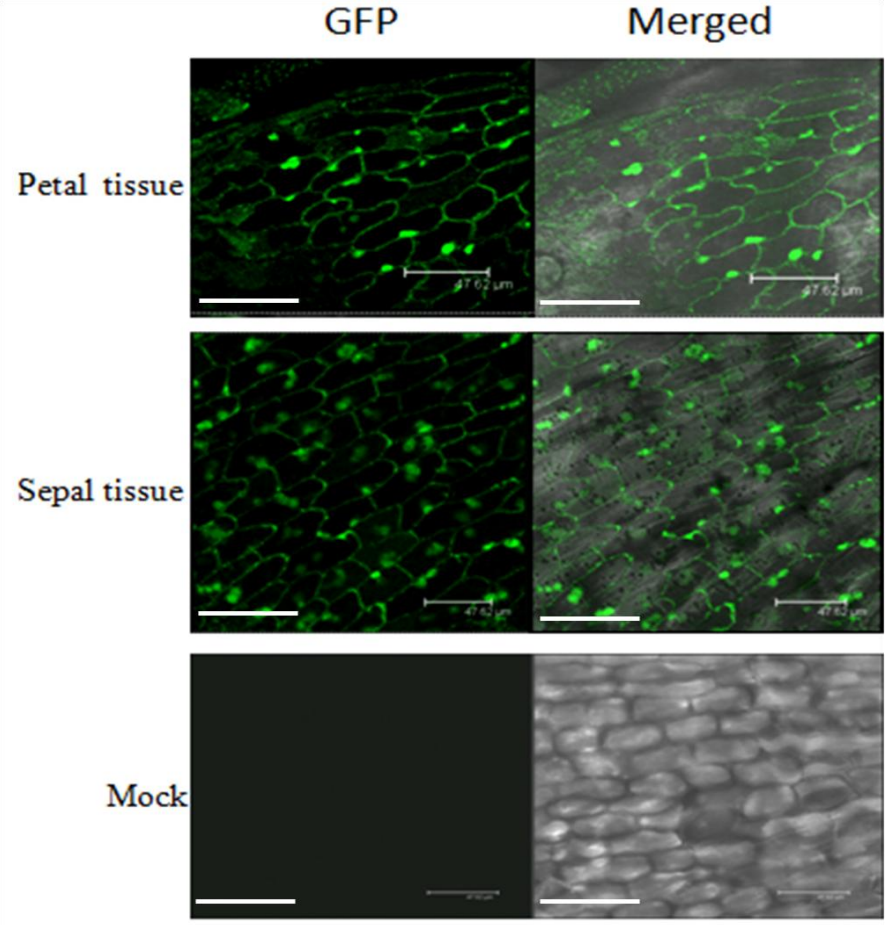

Figure 9: Confocal microscopy of the floral tissues to detect systemic infection. GFP accumulation in the epidermal cells of flower petal (top) and sepal (middle) indicates the presence of SMV in the flower. No GFP signal was detected in the floral tissue from mock-treated plants (Bottom). Scale Bars: 47.62 micron.

(Chen *et al*., 1982). Therefore, RT-PCR, which is a more sensitive detection approach, was also used. Indeed, in addition to the positive floral tissue samples detected by ELISA, some of the ELISA-negative floral tissues were shown to be SMV positive by RT-PCR. As expected, no SMV was detected in the mock tissue samples. RT-PCR analyses of 8 infected plants individually revealed that all tested floral tissues in 3 plants were infected by SMV. The lowest SMV transmission efficiency in the floral tissue was recorded at 43% in one plant (Figure 10).

3.6 Detection of SMV-GFP in seed tissues at R4-R7 stages

Using RT-PCR, SMV was found to be present in all seed tissues including testa, cotyledon and embryo at the development stages (Table 3), though only a relative small percentage (<25%) of tested seed tissues showed SMV-positive (Figure 11).

SMV-infected Williams 82 plants were allowed to undergo pod development (R3 to R4 stages), seed development (R5 to R6 stages), and seed maturation (R7 to R8 stages). Seeds from each stage were collected and immediately processed for GFP visualization. Seeds were dissected into 3 components: seed coat, cotyledon and radicle. SMV infection was confirmed in seed coat tissue at stages R5 and R6 through visualization of GFP (Figure 12, upper and middle panels). However, not all seeds analysed in this study showed consistently positive SMV infection. Nearly 20% of seeds exhibited GFP fluorescence in the seed coat. GFP visualization was also attempted in the seed embryo (radicle). Except for a few seeds, GFP fluorescence was difficult to detect in radical tissues at all three stages (Figure 12, bottom panel). At stages R4 to R7, GFP

accumulation was detected in the epidermal cells of the seed coat and radicle tissue. As expected, GFP fluorescence was not visualized in uninfected seed tissues (Figure 13).

3.7 SMV accumulation in seeds at the reproductive stages R4-R7

SMV accumulation in seed tissues at different reproductive stages was analyzed by quantification of the SMV genomic RNA using qRT-PCR with CP-specific primers. The results showed that SMV viral RNA had accumulated in the seed embryo, but the level of accumulation varied from seed to seed, and from plant to plant (Figure 14). However, the qRT-PCR results also showed that the level of SMV viral RNA was 18-20 folds higher at the R1/R2 stage (Floral tissue stage) than that at the R3/R4 stage (early immature seeds). Interestingly, an increased amount (5-6 folds) of SMV viral RNA was detected at the R6/R7 stage (semi mature stage), in comparison with that at the R3/R4 stage (Figure 14).

3.8 Detection of cell abnormalities induced by SMV

Tissues infected by SMV-L-GFP show characteristic cylindrical inclusions in the cytoplasm. In this study, all infected tissue types were found to contain Type 2 (pinwheel, laminar aggregates) cylindrical inclusion structures in the cytoplasm of infected cells, some of which were closely associated with the rough endoplasmic reticulum (Figure 15). The pinwheel cylindrical inclusions seemed to possess spiral arms.

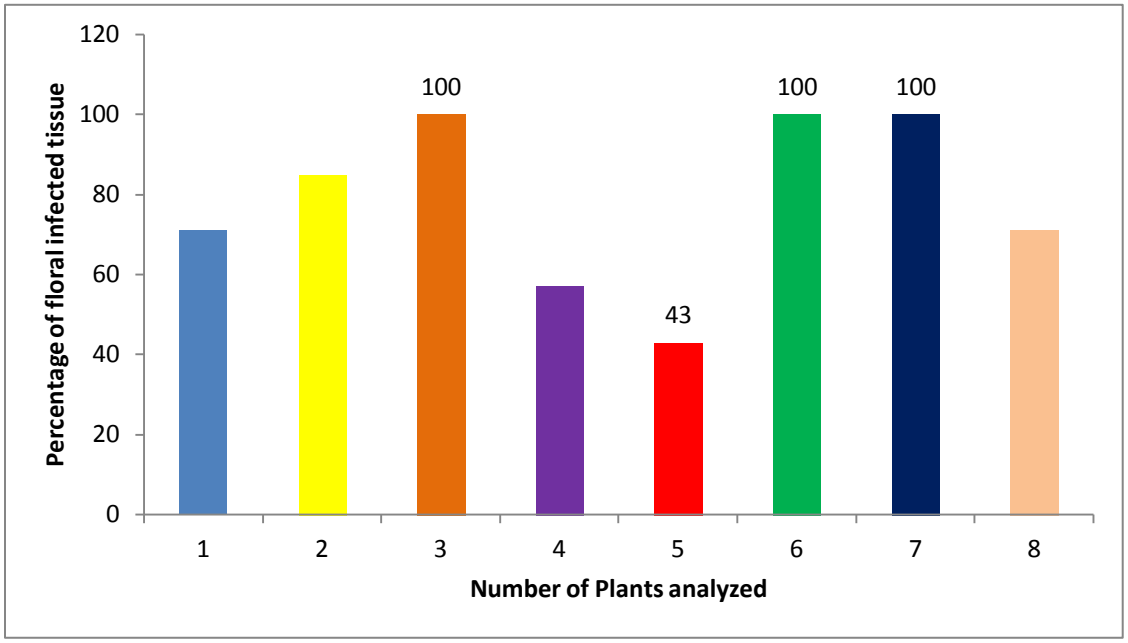

Figure 10: The SMV infection efficiency in floral tissues at the reproductive stage 1 (R1). 100% infection efficiency indicates that all the floral tissues of the three plants (Plant 3, 6 and 7) were SMV-positive. The lowest SMV transmission efficiency in the floral tissue was recorded at 43% in plant 5.

Table 3. RT-PCR detection of SMV in embryonic tissues

Stage	Testa	Cotyledon	Embryo	Percentage* (%)
R4 (Full Pod)	-	4/30		13%
R6 (Full Seed)	17/30	10/30	7/30	33%
R7 (Semi mature seed)	14/30	12/30	5/30	40%
R8 (NM)*(Mature seed)	6/30	9/30	4/30	30%

*Percentage for SMV-positive is calculated on the basis of the number of SMV-positive co+tyledons or embryos over the number of seeds tested. 30 seeds were analyzed for each stage. NM, nonmottled seeds.

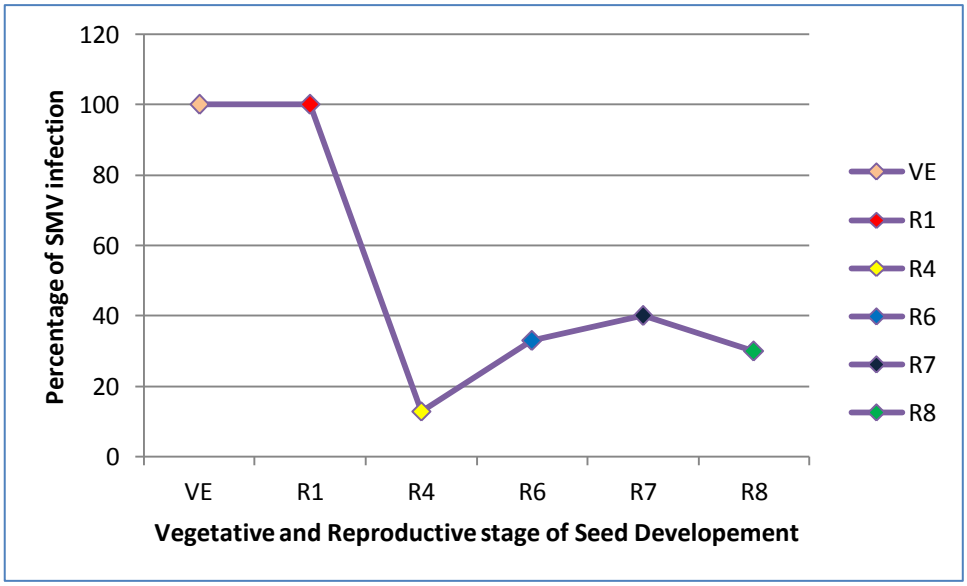

Figure 11: SMV infection efficiency of the vegetative tissue (leaf) and seed tissues at different reproductive stages from Williams 82 plants infected by SMV-L-GFP.

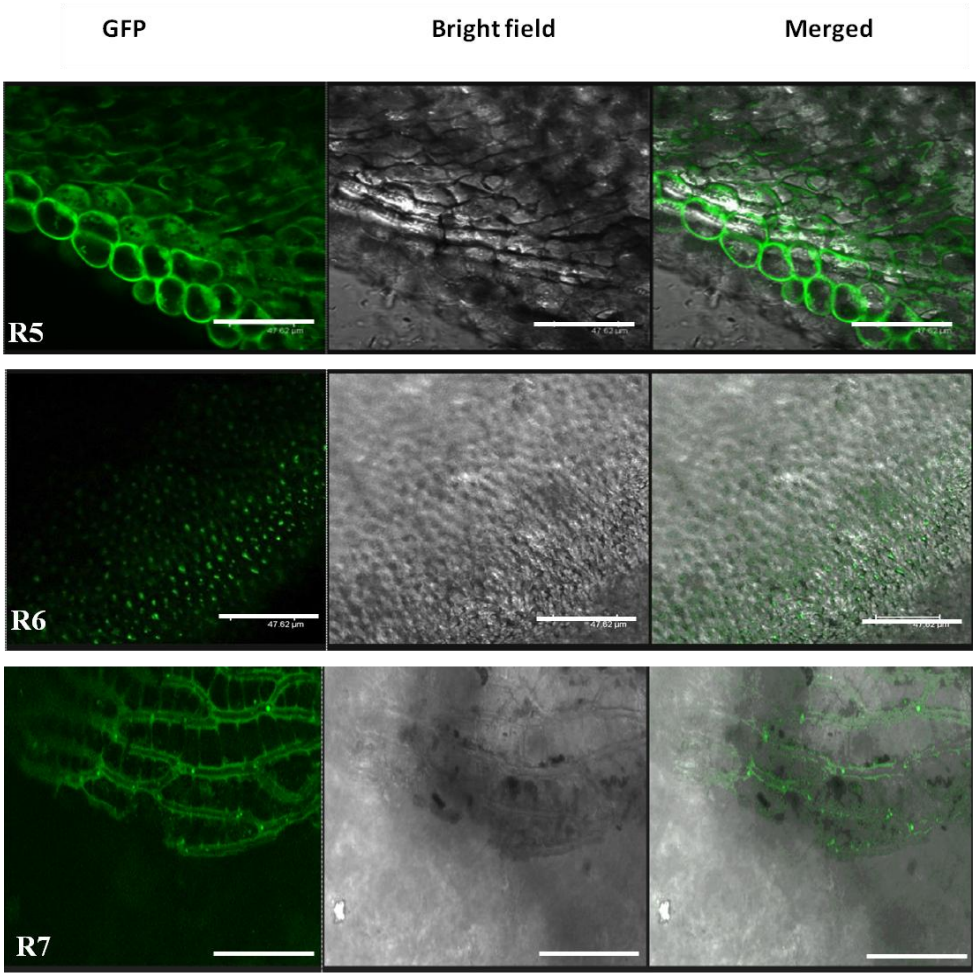

Figure 12: Detection of SMV in seed tissues of Williams 82 infected by SMV-L-GFP at different reproductive stages by confocal microscopy. Upper panel, GFP fluorescence from the seed pod tissue at the R5 stage; middle panel, GFP fluorescence from the seed coat tissue at the R6 stage; bottom panel, GFP fluorescence from the seed radical tissue at the R7 stage. Scale Bars, 47.62 micron.

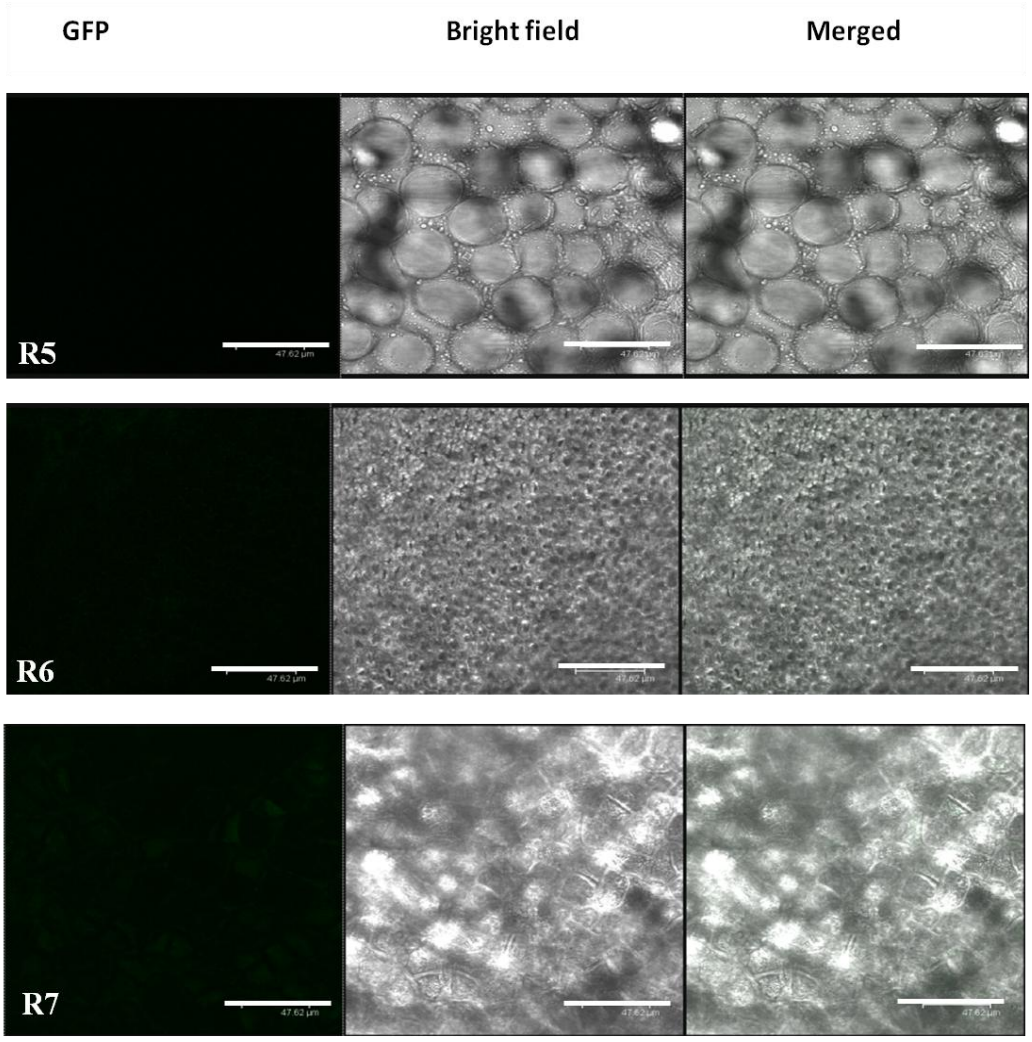

Figure 13: Detection of SMV-GFP distribution in healthy control plants. No GFP fluorescence was monitored from R5-R6-R7 reproductive seed tissue of non-infected plant. Bar size in white at right corner: 47.62 micron.

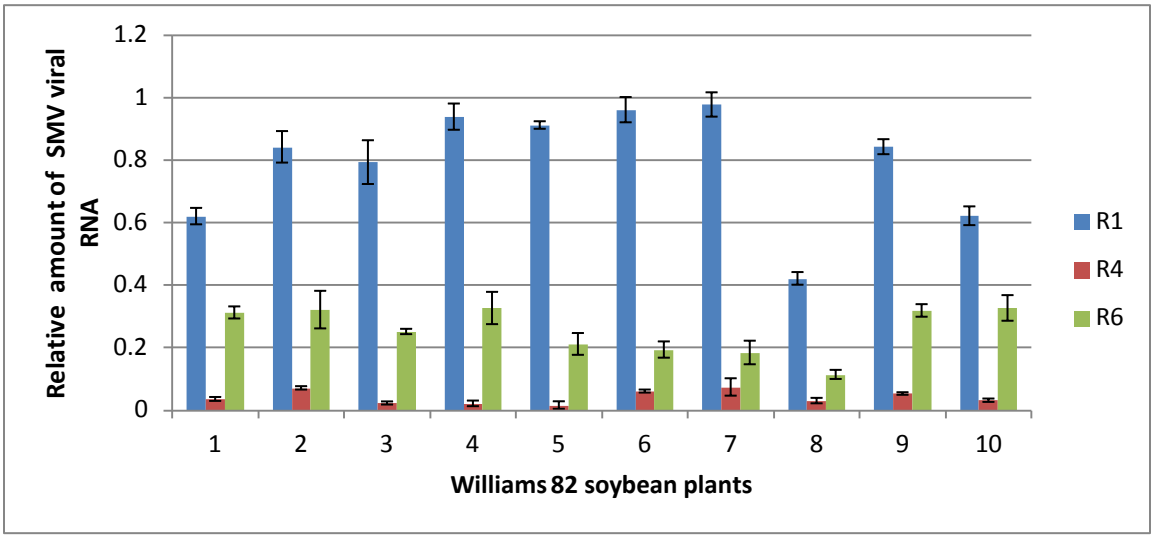

Figure 14: Quantitative real time PCR analysis of SMV genomic RNA. qRT-PCR was performed using CP-specific primers. SMV genomic RNA was highly accumulated at the R1 stage), drastically reduced at R4 (immature embryo stage), and slowly increase when seed reached the semi mature stage and embryo developed fully (R6 stage).

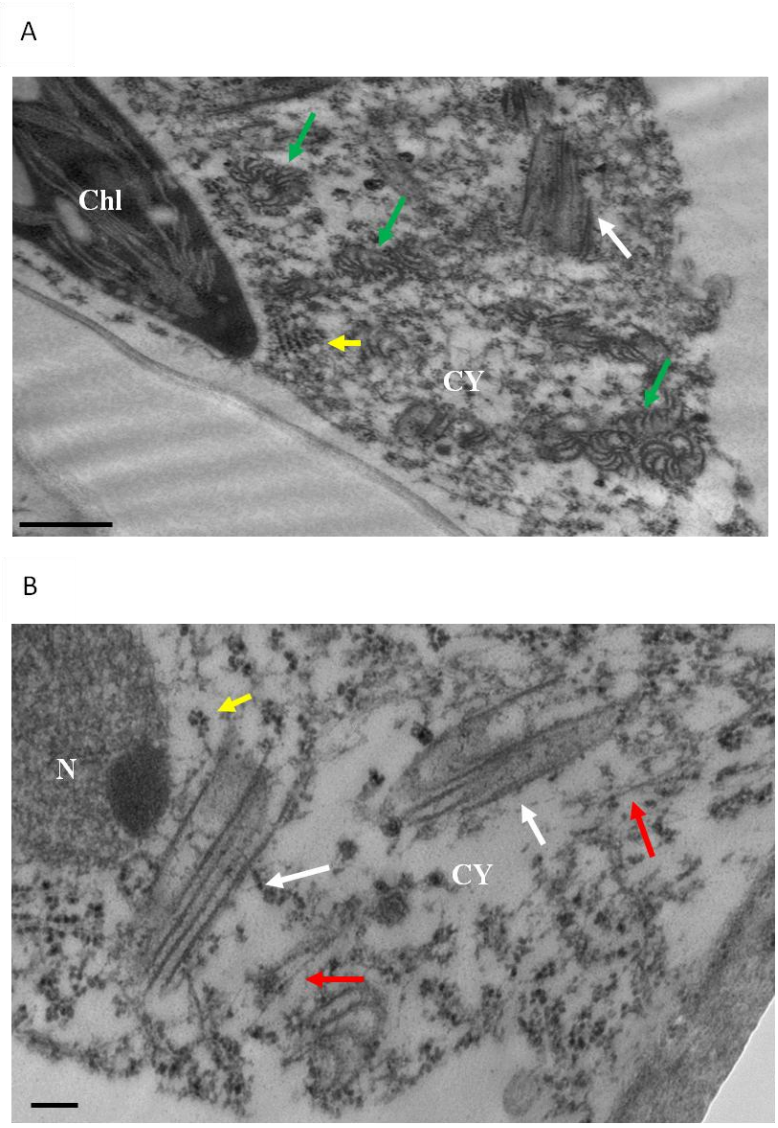

Figure 15: Cytopathology of SMV-infected cells of Leaf tissue. Ultrathin sections of leaf tissue from plants infected by SMV-GFP detected the presence of typical potyviral abnormalities into the infected cell. Note the cylindrical inclusions (White arrows) and flexuous particles (Red arrows) in the cytoplasm, typical of potyviruses. Green arrows indicate the pinwheel inclusions into the cytoplasm. The scale bar at left corner corresponds to 500 nanometer in A and to 100 nanometer in B. Chl, Chloroplast, CY, Cytoplasm. Yellow arrow indicates Rough Endoplasmic Reticulum (RER).

Seeds at the R3 and R4 stages were also analyzed for detection of SMV induced cell abnormalities. Interestingly, cytopathology of the seed embryo also confirmed the presence of spiral pinwheel cylindrical inclusion-type structures in the cytoplasm, but much closer to the plasmodasmata region (Figure 16).

3.9 Probe synthesis for *In situ* hybridization

To generate a DIG-labeled, sense, single stranded RNA (ssRNA) probe spefically for detection of the negative SMV RNA, SMV cDNA was applied using the CP-specific primers. The resulting PCR product was futher used, via PCR, to engineer a T7 polymerase priming site for directional *in vitro* transcripton. Generation of DIG-labeled, sense, single stranded RNA (ssRNA) probes from DNA templates by *in vitro* transcription with T7 RNA polymerase was successful and detection of the probes with anti-DIG-AP and NBT/BCIP was possible as shown through a dot blot (Figure 17).

3.10 Detection of the negative sense SMV RNA in seed tissues by ISH

To identify cells supporting active virus replication, sections of SMV-infected immature seeds were probed for the negative sense SMV RNA with the use of *in situ* hybridization. Since the negative sense viral RNA is considered to be the replicative form of the viral RNA, *in situ* hybridization was used to detect the negative sense RNA of SMV in seed tissues. The current study not only showed the abundance of RNA accumulation in the epidermal outer and inner integument of the seed, but also provided evidence of RNA accumulation in the suspensor base region that connects to the embryo (Figure 18). These

data suggest that the infected suspensor base region provides a route for virus invasion into the embryo. However, many immature seeds had RNA accumulation outside the embryo and suspensor base regions (Figure 18, bottom panel). In the infected areas, there was a uniform accumulation of viral RNA and a sharp differentiation between infected and uninfected areas, which suggests that SMV replication is rapid (Figure 18). As expected, no RNA signal was found in the nonseed transmitted seed embryo (Figure 19). To make sure that the RNA probe properly hybridized with the negative sense RNA, the same probe was also applied to young, infected and healthy leaf tissue samples. As expected, negative sense viral RNA accumulation was detected in the epidermal cells of infected, young leaf tissue samples but no RNA accumulation was observed in the healthy tissue samples (Figure 20). The probe was also applied to the infected floral and seed coat tissues. Viral RNA accumulation was observed in the epidermal cell of the tissues. However, this problem was only limited to R3 and R4 stages of seed, as this is the stage when the embryo starts to develop and remains connected to the suspensor region.

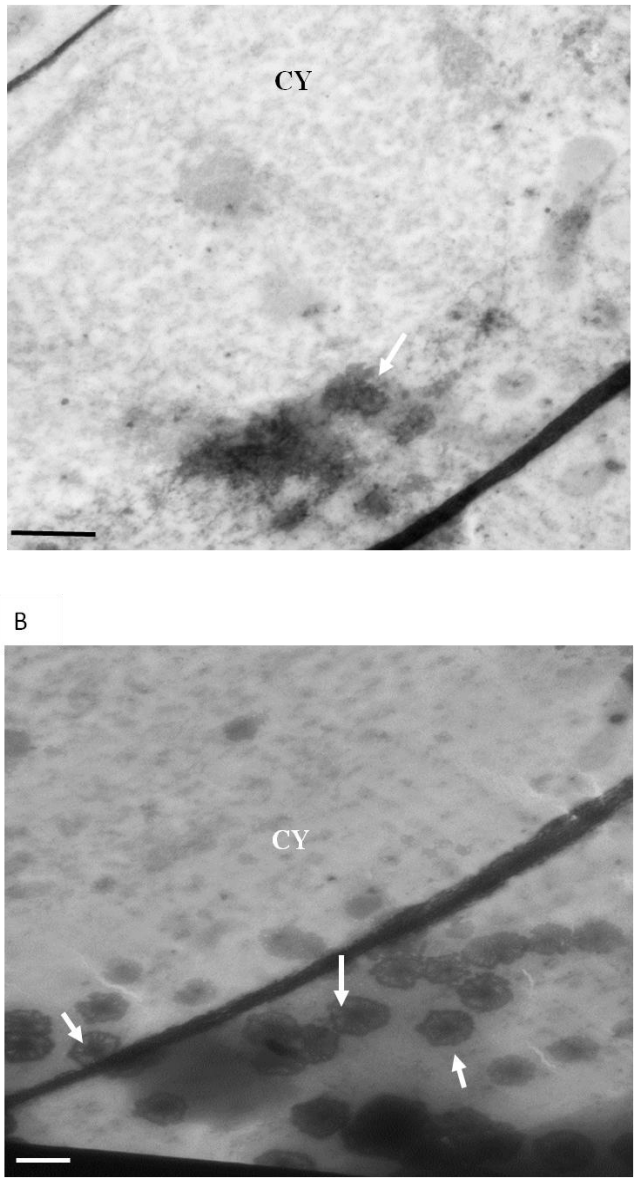

Figure 16: Cytopathology of SMV-infected cells of R3/R4 reproductive seed embryo. Ultrathin sections of embryo tissue from plants infected by SMV-GFP detected the presence of typical cylindrical inclusions (white arrows) in the cytoplasm. Cylindrical inclusions are mostly located close to plasmodesmata. The scale bar corresponds to 500 nm in A and to 100 nm in B. CY, Cytoplasm.

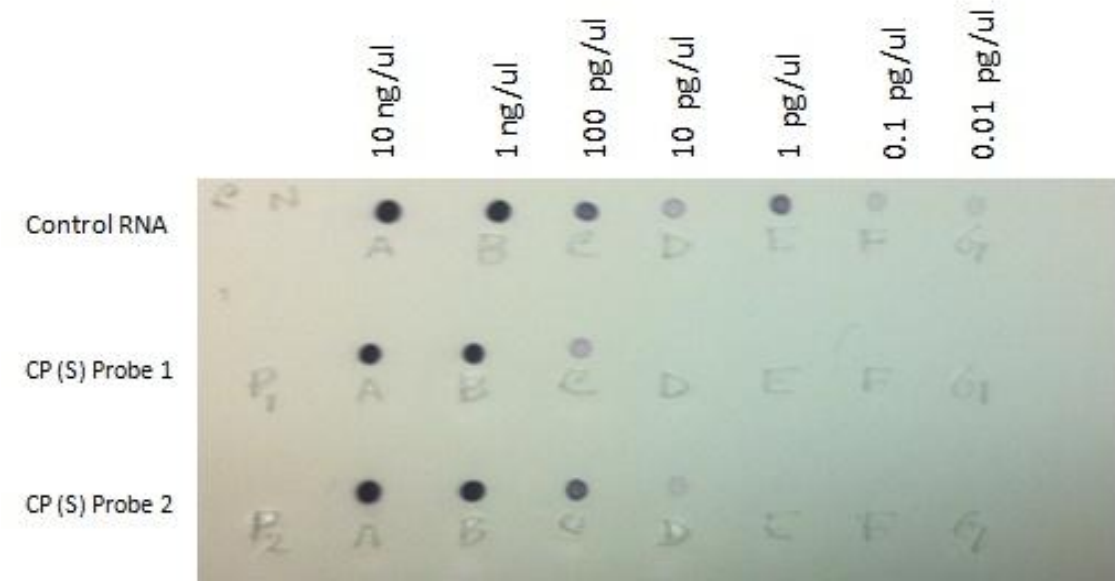

Figure 17: Spot blots of DIG-labeled probes for *in situ* hybridizations. 1 µL dilutions of the DIG-labeled probes were spotted onto positively charged Nylon membrane. The serial dilution factors of the sense (S) probes were (from left to right); 1:10, 1:100, 1:1X10^3, 1:1 X10^4, 1:1 X10^5, 1:4 X10^6, 1:1X10^7. 1 µL volumes of serial dilutions of control labeled RNA (C) were also spotted alongside each probe for estimation of quantity of DIG-labeled probes by comparison of spot intensities following detection with anti-DIG-AP fragments and coloring reaction using NBT-BCIP.

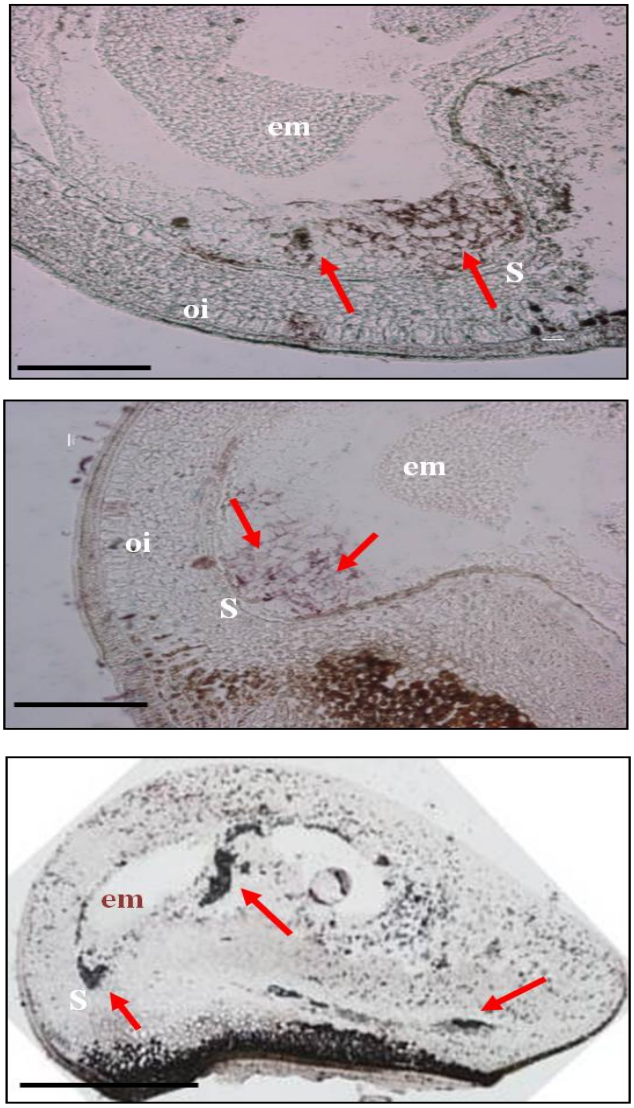

Figure 18: *In situ* hybridization of the negative sense SMV RNA in the seed embryo. Sections of seed immature (R3/R4 stage) embryos were hybridized with a digoxygenin-labeled RNA probe to specifically detect the CP region of the negative sense SMV RNA and viewed under a compound microscope (bright field). Purple or dark brown color indicates positive hybridization. em, embryo; S, suspensor base region; oi, outer integument region. Scale bar, 500 micron (upper and middle panel) and 1 millimeter (bottom panel). Red arrow indicates signal accumulation.

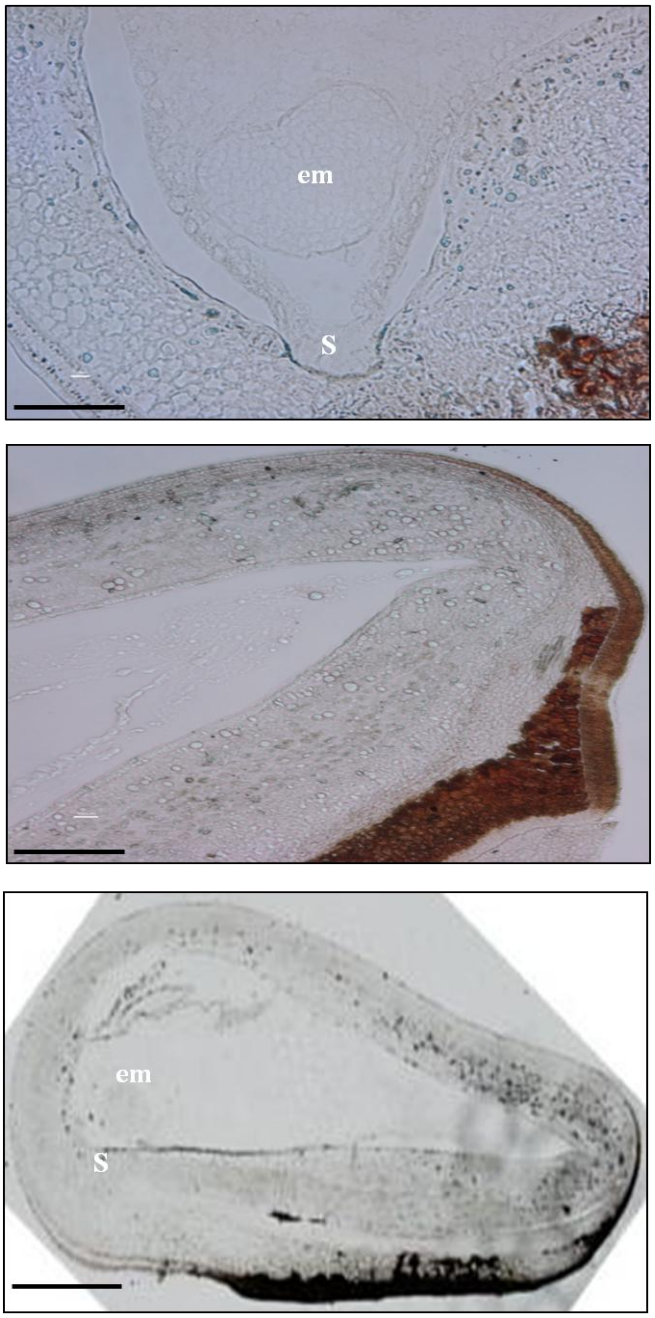

Figure 19: *In situ* hybridization of the negative sense SMV RNA in non seed transmitted seed embryo by CP sense probe. No viral accumulation detected within the embryo or suspensor region. Black scale bar, 500 micron (Upper and middle panel) and 1 millimeter (Bottom panel). S, Suspensor base region; em, embryo.

Healthy leaf

Infected leaf

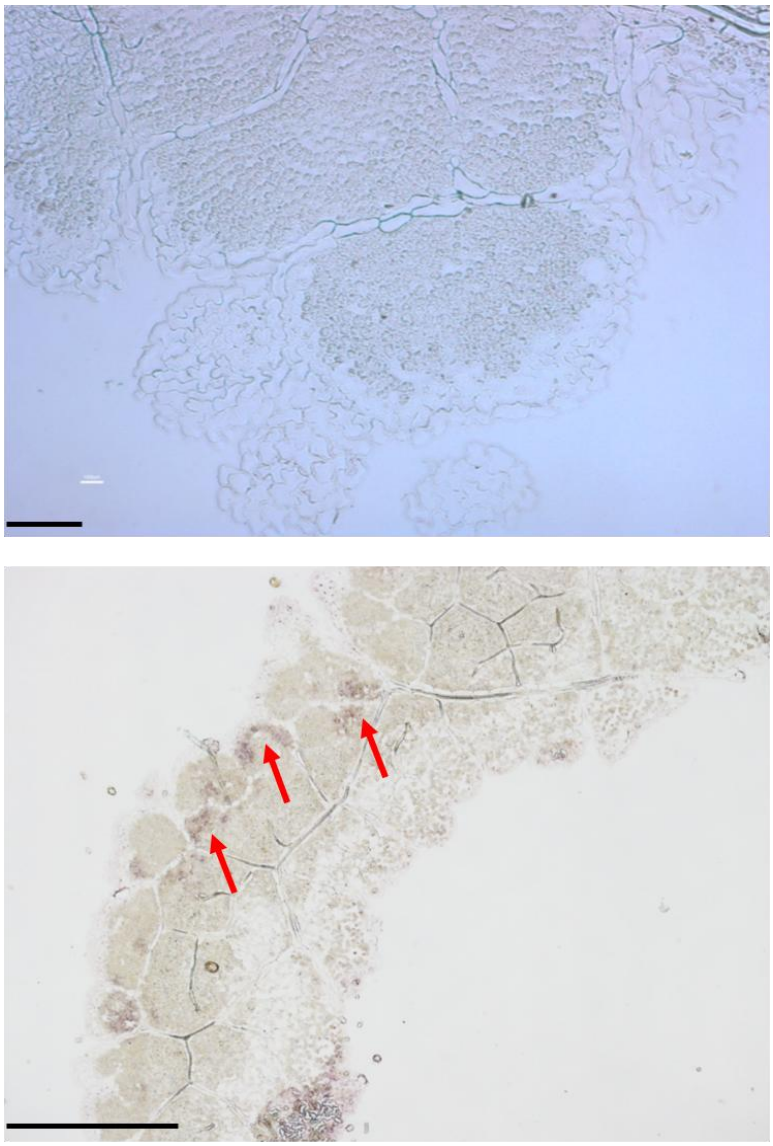

Figure 20: *in situ* hybridization of negative sense CP transcript detection in infected and healthy leaf by CP sense probe. No viral accumulation detected in the healthy leaf (Upper panel, Scale bar, 100 micron), whereas detection of purple spot detected in the epidermal cell of infected leaf indicated by red arrow (Bottom panel, Scale bar, 1 millimetre).

3.11 Detection of dsRNA in the seed tissue by immunolocalization

As the formation of dsRNA is a hallmark of the viral replication process, the sections of the seed tissue were probed by monoclonal dsRNA-specific mouse antibody J2, which specifically recognizes dsRNA provided that helix length is greater than or equal to 40 bp (Lukacs, 1997). Results showed that J2 specifically detected dsRNA as a strong fluorescence signal only in infected leaf epidermal tissues (Figure 21). No positive signal was detected in the corresponding leaf tissues from healthy plants (Figure 21). These data suggest that this system works well to detect SMV replicative dsRNA.

In order to further confirm that SMV replicates in the embryo, the sections of seed embryos were incubated with J2 antibody for the detection of dsRNA. Strong fluorescent signals were evident in the inner and outer integument regions, and less intensive but clear fluorescent signals were observed in the embryo, suspensor and suspensor base regions (Figure 22), suggesting SMV replication does occur in soybean embryos. These data are consistent with the observation that the seed coats developed from the inner and outer integument region accumulate with higher levels of SMV than the seed embryo. In addition, fluorescent signals were present in the zone that connects with the embryo, and this was also corroborated by *in situ* hybridization assays. To rule out the possibility that this signal is not a false positive, seeds from healthy plants and were also used as a negative control. As expected, no fluorescent signal was found in healthy seed embryos (Figure 23).

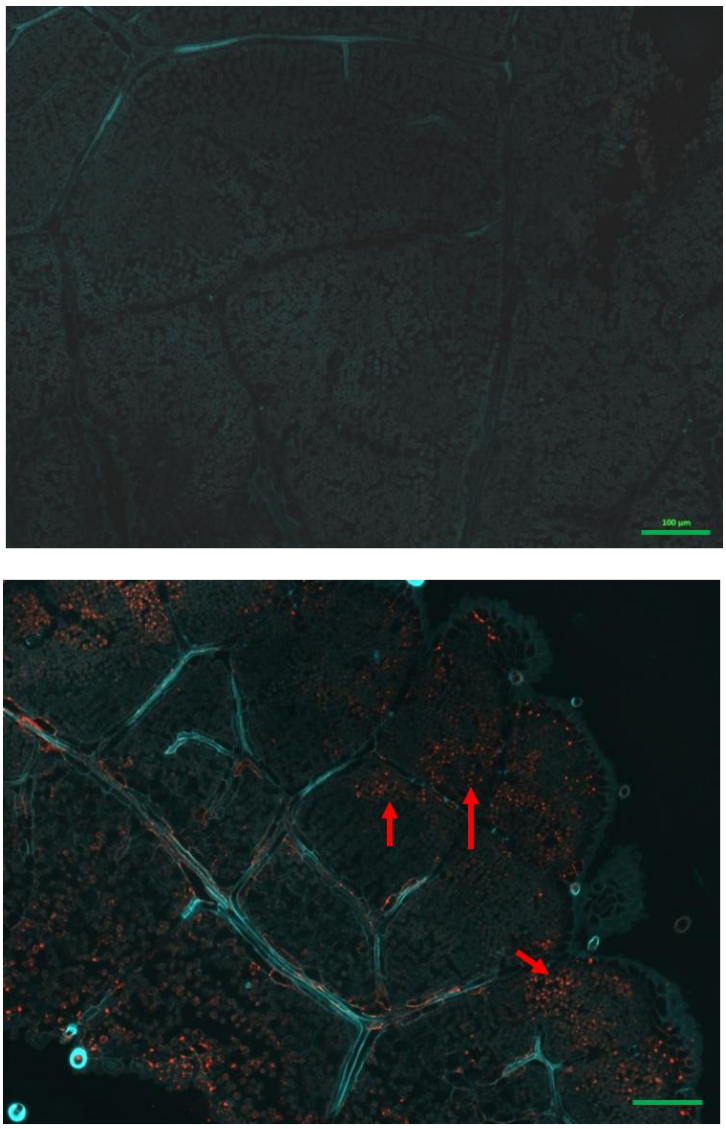

Figure 21: dsRNA detection by mouse monoclonal J2 antibody in the infected and healthy leaf of Williams 82 plants. dsRNA fluorescence indicates by red color. No dsRNA signal detected in the healthy leaf (upper panel), whereas red fluorescence was clearly detected in the epidermal cells of the infected leaf indicated by red arrows (bottom panel). Scale bar, 100 micron.

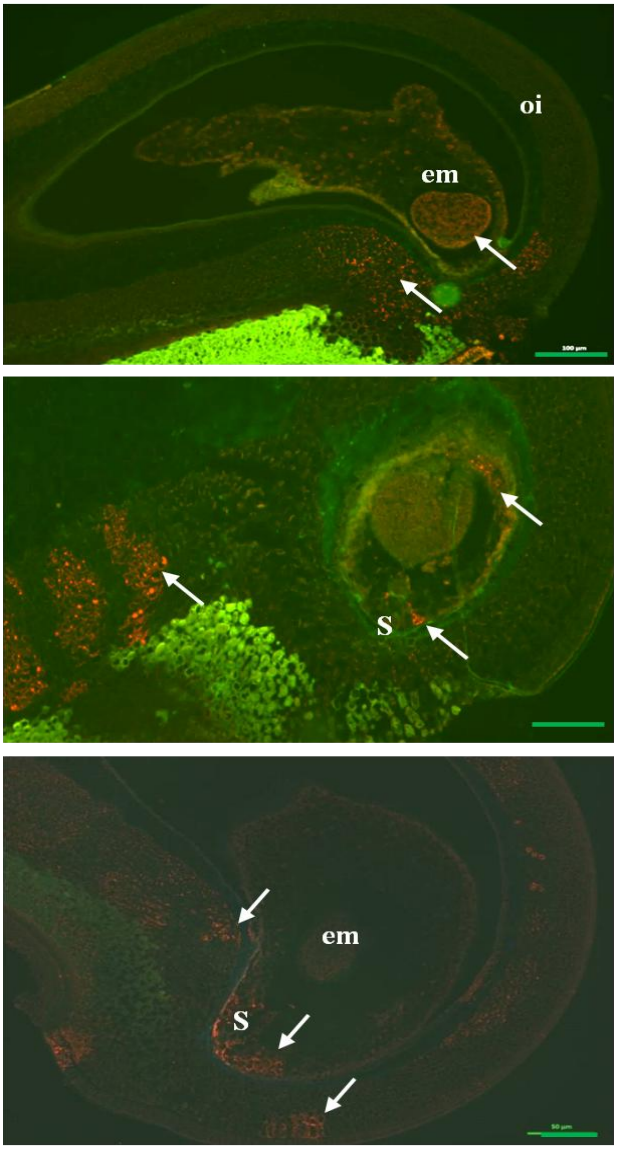

Figure 22: dsRNA detection by mouse monoclonal J2 antibody in the immature R3/R4 stage seed embryo. Seeds were micro sectioned with the wax microtome and J2 mouse monoclonal antibody used to detect dsRNA fluorescence in the seed embryo. dsRNA fluorescence was detected in both the suspensor and embryo region of three individual seeds. Scale bar, Upper panel: 100 micron, middle panel: 50 micron and bottom panel: 50 micron. em, embryo, S, suspensor base region, oi, outer integument region.

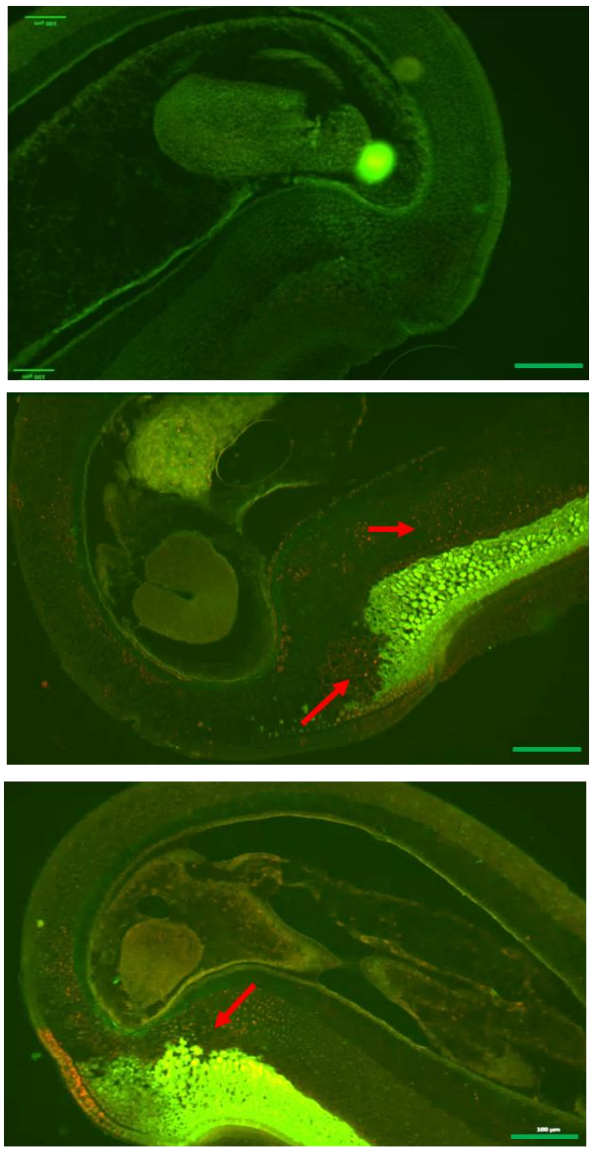

Figure 23: dsRNA detection by mouse monoclonal J2 antibody in the healthy and infected seed embryo. No red fluorescence detected within the embryo or other regions in the healthy control (top panel). Mild fluorescence detected in the outer integument region in the sample from an SMV-infected seed. Red arrows point to cells stained by red fluorescence. Scale bar, 50 micron (upper and middle panel), 100 micron (bottom panel).

3.12 Distribution of SMV in mature seed tissues

To understand the distribution of SMV in mature seed tissues, mottled mature seeds harvested from SMV-infected plants were soaked in DEPEC treated water at room temperature for germination in dark. When seeds became swollen and radicle embryonic shoots emerged, seeds were separated into three components, i.e., seed coat, radicle and cotyledon (Figure 24A). Each of these components was used for detection of SMV in different parts of the seed tissue by ELISA and RT-PCR. A total of 80 seeds at the R8 stage from infected Williams 82 plants were analyzed. ELISA revealed SMV in 20%, 15% and 26% of the seed coat, radicle and cotyledon, respectively, whereas RT-PCR was more sensitive with higher SMV distribution efficiency (23%, 18% and 33%, respectively) in the corresponding seed tissues (Figure 24B and C). These results indicate that although SMV distributed in the seed testa, cotyledon and radical tissue, its transmission efficiency varied among seed tissues.

Nonmottled seeds harvested from SMV-infected soybean plants were also plated for germination under similar conditions. Following germination and subsequent growth to the cotyledon stage (day 4), radicle shoots were sectioned immediately for GFP visualization by confocal microscopy. RNA extraction was also performed using radical tissues. A total of 30 nonmottled seeds were analyzed. RT-PCR results revealed that only 13% of radicle tissues were infected by SMV. However, when the same tissue was analyzed for GFP visualization by confocal microscopy, no GFP signal was detected in tissue samples which had been found to be SMV-positive by RT-PCR.

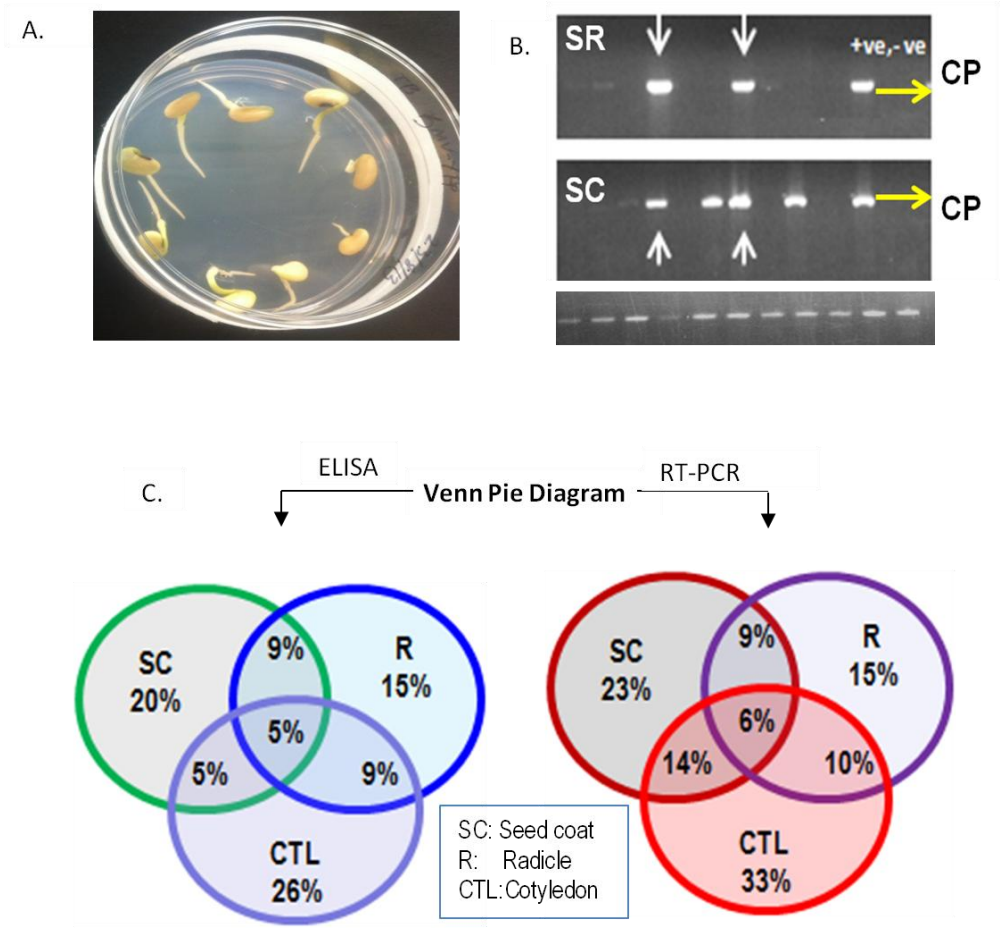

Figure 24: SMV distribution in different seed tissues. Seeds were placed in Petri dishes containing MS Medium for germination at dark for 2-3 days. Black arrow indicates an embryo radical (A). The presence of SMV in SR (Seed Radicle) and SC (Seed Coat) was detected by RT-PCR. White arrows indicate different tissues from the same seed were SMV-positive (B).Venn Pie Diagram shows ELISA revealed SMV in 20%, 15% and 26% the seed coat, radicle and cotyledon, respectively, whereas RT-PCR confirmed 23%, 18% and 33% of SMV distribution rates, respectively (Right) (C). A total of 80 seeds at the R8 stage from infected Williams 82 plants were analysed.

3.13 Efficiency of SMV transmission in harvested seed from infected plants

A total of 120 infected mottled seeds and 90 infected non-mottled seeds harvested from SMV-infected Williams 82 plants were planted in a growth chamber. SMV was found in the seeedlings from both mottled and nonmottled seeds. Infected leaf tissue samples showed typical chlorosis and mosaic symptoms, whereas healthy plants did not exhibit any symptom development (Figure 25). As shown in Figure 26, mottled seeds showed a higher efficiency of SMV transmission (15% to 26%) than non-mottled seed (0% to 14%). These data suggest that SMV can be transmitted in either mottle or nonmottled seeds.

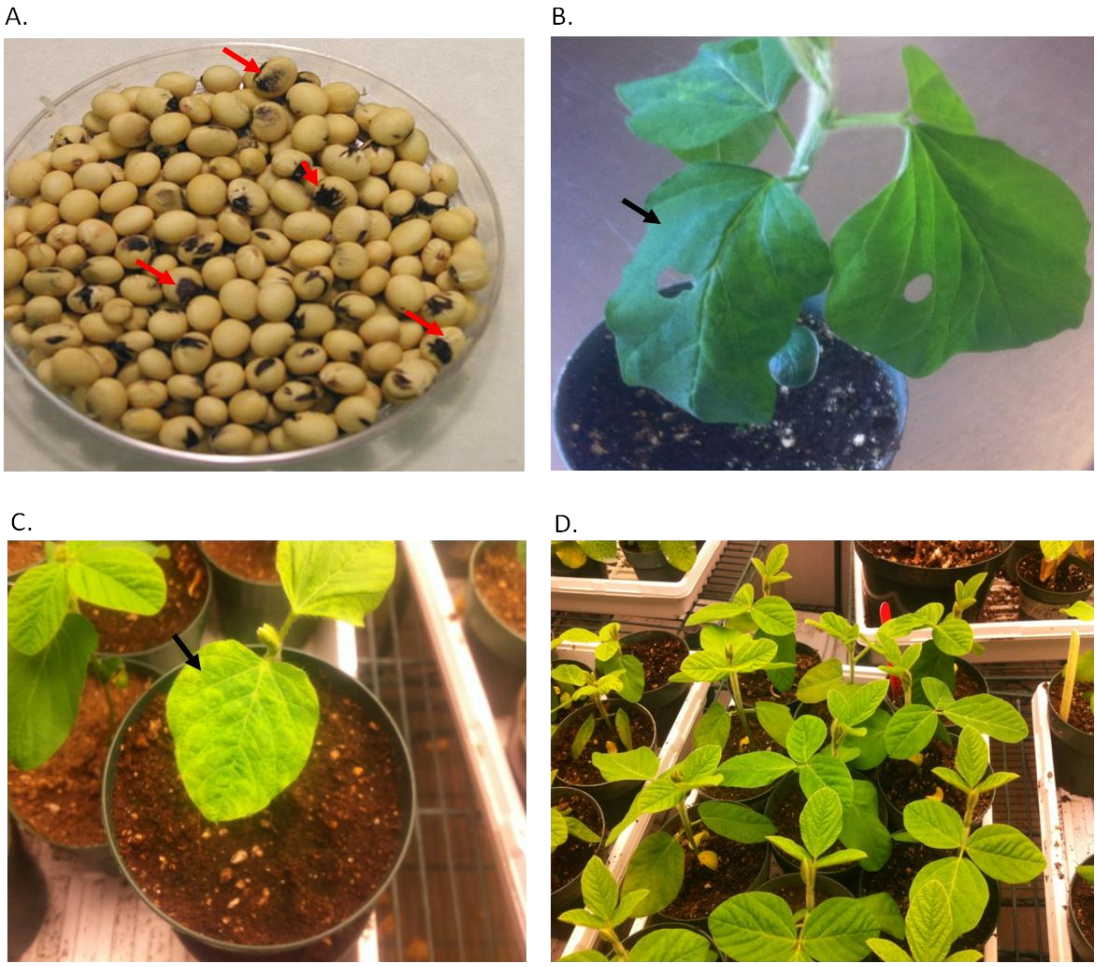

Figure 25: SMV transmission during seedling. Mottled seeds (red arrows) from SMV-infected plants were selected for germination (A). Three-week-seedlings were observed for SMV symptoms. About 20% seedlings showed symptoms (back arrows) such as mosaic (B) and chlorosis (C) in the trifoliate and unifolate leaf. About 80% seedlings appeared asymptomatic or healthy (D).

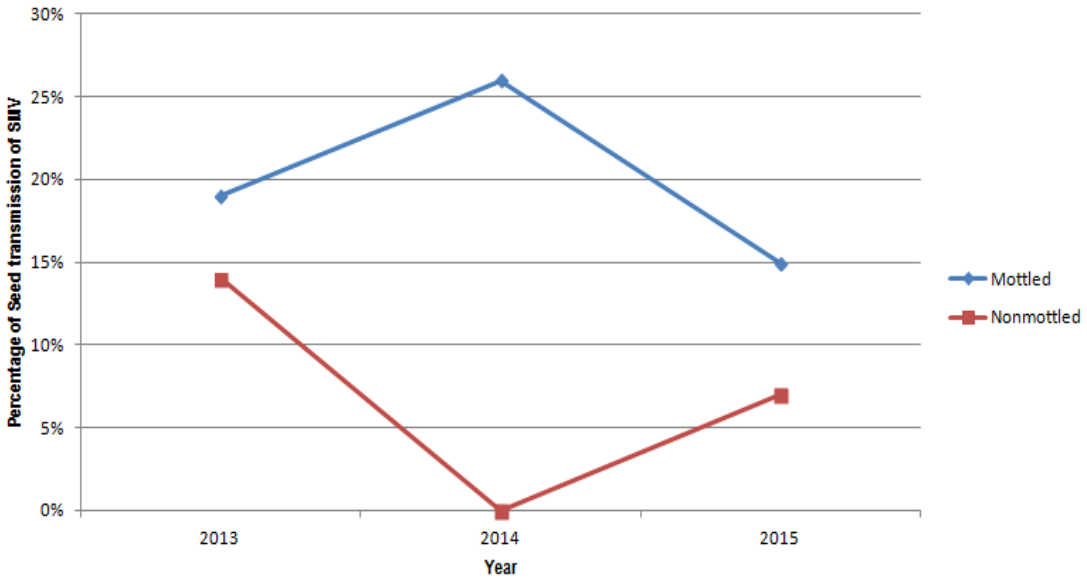

Figure 26: Percentage of SMV-positive seedlings geminated from mottled and non-mottled seeds of SMV-infected plants. Mottled and non-mottled seeds were used for SMV transmission from 2013 to 2015. A total of 120 mottled seeds and 90 nonmottled seeds were analyzed in this study. Mottled seeds showed a higher efficiency of SMV transmission (15% to 26%) than non-mottled seed (0% to 14%).

4 DISCUSSION

4.1 Research overview

Viral seed transmission is an inherent property through which a potential seed transmitted virus infects its host during the plant reproductive stage, and establishes itself in the embryo to infect the progeny (Wang and Maule, 1992). It is assumed that approximately 20% of plant viruses are transmitted from generation to generation through seed (Matthews, 1991; Wang and Maule 1994). So far, little progress has been made in elucidating the molecular mechanisms of seed transmission. It is not known, for example, how the virus moves from sporophytic to gametophytic tissues, and back to sporophytic tissue, or from the infected maternal testa to the embryo. The extent of seed transmission depends on the cultivar, the virus isolate and the time of infection (Mumford, 2006). The current study focuses on one of the major seed transmitted viruses, SMV. It is a notorious viral pathogen of legumes, with soybean as the primary agronomic host (Hill, 1999; Hobbs *et al*., 2003; Gunduz *et al*., 2004). In addition to seed transmission, SMV spreads efficiently by pollen, mechanical inoculation, and aphids in a non-persistent manner (Arif and Hassan, 2002). In North America, SMV rarely infects alternative host species and seed-borne infections are the primary sources of inoculum (Domier *et al*., 2011). Blocking seed transmission is an unexplored method to control SMV infection. But, seed transmission efficiency, and the mechanisms behind seed transmission, in soybean cultivars are poorly understood. So, the present study was designed to attempt to characterize seed transmission of SMV in soybean.

4.2 SMV transmission and replication in soybean seeds

My first hypothesis was that SMV transmission in seeds proceeds via the infected maternal testa into the embryo. To investigate this, I used a GFP-tagged infectious clone (SMV-L-GFP) that was previously constructed in the Wang lab. This clone can efficiently infect the Williams 82 soybean cultivar that does not carry SMV resistance gene but cannot infect PI 96983 and PI 596752, both of which carry a resistance gene. Williams 82 soybean plants infected by SMV-L-GFP were closely monitored for the presence of SMV in vegetative and seed tissues at different reproductive stages by a combination of DAS-ELISA, RT-PCR and confocal microscopy techniques. To the best of our knowledge, this was the first attempt to use confocal microscopy for to confirm and monitor SMV infection in soybean vegetative and seed tissues. As expected, Williams 82 showed typical symptom development such as mosaic patterning, mottling and leaf chlorosis, and leaf wrinkling, while plants carrying *Rsv*1 or *Rsv*4 resistance loci showed no symptom development (Figure 4 and 5). The infected Williams 82 plants were further analyzed for seed transmission.

Infection in the embryo appears to be a significant factor affecting seed transmissibility of plant viruses (Sastry, 2013). Embryo infection can occur after fertilization via direct invasion of the seed tissues, or indirectly before fertilization through infection of the gametes (Maule and Wang, 1996). As infection of plants before flowering can lead to embryonic infection (Sastry, 2013), floral tissues were examined for the presence of SMV-L-GFP by confocal microscopy, and further tested by ELISA and RT-PCR. Interestingly, only 3 out of 8 infected Williams 82 plants showed 100% infection, while the remaining 5 plants showed 43-85% infection in floral tissues (Figure 10). It is not

clear why the virus was unable to infect 100% of the floral tissues, as all the plants were from the same cultivar and were infected with the same virus. Disruption of viral transmission to the floral tissue in the infected plants, due to cytoplasmic separation of developing floral tissue from maternal tissue, or the inability of the virus to invade the ovule might explain the above phenomenon. Nevertheless, it can be concluded that SMV had access to floral tissues, as almost all infected floral tissues showed GFP fluorescence in epidermal cells (Figure 9).

I further detected SMV in the embryo at all the reproductive stages of seed development, although the degree of infection varied from plant to plant (Table 3). These data confirm that SMV seed transmission occurs via embryo infection. In addition to the embryo, the virus was also found in the testa and cotyledon of immature and mature seeds (Figure 12). Moreover, the extent of SMV infection was high in the floral tissue at the R1 stage, apparently reduced at the R4 stage, but eventually maintained at the relatively higher level (than that at R4) for the rest of the reproductive stages examined (Table 3, Figure 11). To determine if this phenomenon was related to viral accumulation, the relative amount of SMV viral RNA at the different reproductive stages were quantified by qRT-PCR. As shown in Figure 14, the level of SMV viral RNA was indeed at the highest level at the R1 stage, lowest (about 10 times) at the R4 stage and high again at the R6 stage to about half of that at the R1 stage. These data indicate that R4 is a critical stage for SMV seed transmission, consistent with the previous finding that SMV transmission was apparently inhibited after the seed pod development stage (Bowers, 1979).

In this study, although SMV was present in the testa, cotyledon and embryo of both immature and mature seeds, visualization of GFP fluorescence was very difficult in all

infected seed tissues. The inability to detect GFP fluorescence in seed tissues at reproductive stages R4 to R7 is not in agreement with RT-PCR results. This is possibly because very low levels of translation occur in seed tissues during seed maturation and SMV becomes dormant.

In this study, I detect SMV infection in different tissues of both mottled and non-mottled seeds (Figure 25A), consistent with previous findings (Pacumbaba, 1995; Bajwa and Pacumbaba, 1996). However, seeds that displayed mottling typical SMV infection transmitted SMV into the next generation much more efficiently than non-mottled seeds (Figure 26).

The potyvirus CI protein is a multifunctional protein that plays a pivotal role in virus replication as well as long distance and local virus movement (Sorel *et al*., 2014; Ivanov *et al*., 2014). For potyviruses, translation and replication of viral genomes are a coupled process (Wang 2016, Annual Review of Phytopathology in press). In newly infected cells, some of the CI proteins, upon translation, attach to the plasmodesmal apertures through which the virus can pass to the adjacent cell. This happens only during active virus replication in the cell, after which the CI proteins disassociate from the cell wall, accumulate in the cytoplasm, and begin to degenerate (Shukla *et al.*, 1994; Roberts *et al*., 1998). The current study also examined if SMV infection results in characteristic cylindrical inclusion structures in infected cells. Cytopathological investigation of the leaf and embryo tissues infected by SMV-L-GFP showed the presence of typical potyviral cylindrical inclusion structure in the cytoplasm and near the boundary of the cell wall (Figure 15 and 16). This is in agreement with previous findings showing pinwheel aggregates of CI in the cytoplasm of infected cells (Wang and Maule 1994;

Roberts *et al.*, 1998; Roberts *et al.*, 2003; Ivanov et *al*., 2014). My study represents the first report that CI structures are present in the embryogenic cells as pinwheel aggregates (Figure 16). Taken together, these results suggested that SMV infects the embryo and can multiply itself and spreads therein.

4.3 SMV invasion into the embryo via the suspensor

To further confirm SMV replication in soybean seeds, I detected and localized negative strand viral RNA, as well as double stranded RNA (dsRNA) in SMV-L-GFP-infected tissues. For detection of the negative sense of SMV viral RNA, a 421 bp sense probe of the CP gene was prepared and optimized in the leaf tissue. The probe detected the accumulation of the negative sense RNA in the cell cytoplasm in SMV-infected leaf tissues. The probe also clearly detected the accumulation of the negative sense RNA in the embryonic cells, the outer integument region, the suspensor base and suspensor regions of the seed that connect the SMV-infected maternal tissues with the embryo (Figure 18). dsRNA is a signature of viral replication. An immunofluorescence assay using dsRNA-specific J2 mouse monoclonal antibody was also used to localize SMV replication in seed tissues. J2 has been used successfully in a number of dsRNA detection experiments, particularly for viral dsRNA (Wei *et al*., 2010; Bamunusinghe *et al*., 2011; Choi *et al*., 2012; Barajas *et al*., 2014; Kaido *et al*., 2014). As shown in Figure 22, J2 successfully detected the production of SMV-induced dsRNA in the suspensor, embryo and outer integument region, highly consistent with the results of *in situ* hybridization. In a previous study, Wang and colleagues (1994) used ISH techniques for detection of the

seed-borne and seed transmitted *Pea seed-borne mosaic virus* (PSbMV) genomic RNA in the seed embryo of pea and found that PSbMV used the embryonic suspensor for direct invasion of the embryo (Wang and Maule, 1994; Roberts *et al*., 2003). It seems that SMV and PSbMV have evolved to use similar routes to infect the seed embryo for seed transmission.

Based on the results of *in situ* hybridization and dsRNA immunofluorescense experiments, I propose a model of the direct invasion of SMV into the embryo (Figure 27). In this model, SMV invades the ovule wall. In later developmental stages of the seed and the embryo, SMV reaches the micropyle region and subsequently the interface between the testa and suspensor cells. Before the suspensor undergoes its programmed degeneration, SMV passes through the suspensor to enter to the embryo following this sink to source path. Within the infected embryo cell, the virus replicates and moves into neighbouring cells, either by plasmodesmata or embryo sac fluid, before seed maturation. The deficit of this model is that there lacks direct evidence to prove the symplastic pathway of virus movement in the short-lived suspensor cells.

4.4 SMV replication during germination and seedling stages

After SMV-infected soybean seeds break dormancy and germinate, the virus must reactivate its replication during seedling stage to establish infection. To explore when and where SMV starts replication during seed germination and early seedling stages, germinating soybean seeds were dissected into various tissue components.

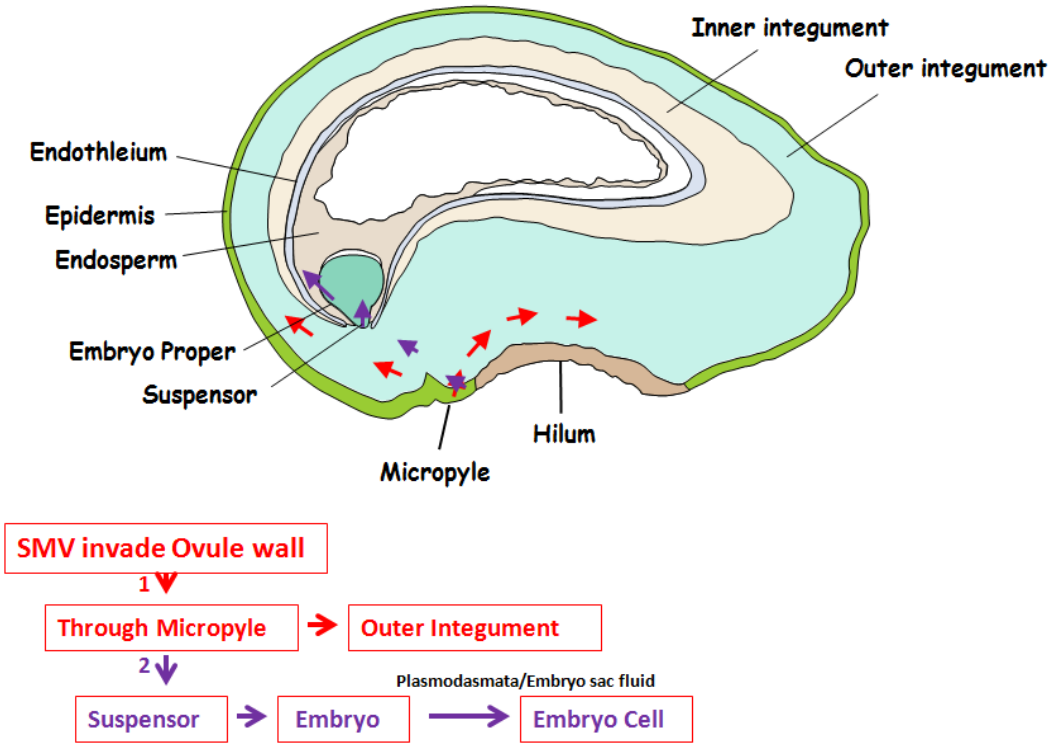

Figure 27: Proposed model of embryo invasion by SMV. It is proposed that, SMV initially invades the ovule wall. In later developmental stages of the seed and the embryo, SMV reaches the micropyle region and subsequently the interface between the testa and suspensor cells. Before the suspensor undergoes its programmed degeneration, SMV passes through the suspensor to enter to the embryo. Within the infected embryo cell, the virus replicates and moves into neighbouring cells, either by plasmodesmata or embryo sac fluid, before seed maturation.

GFP signals were not detected in the different seed parts by confocal microscopy; however, both ELISA and RT-PCR readily detected SMV in the seed coat, cotyledon or radical tissues (Figure 24 B and C). This result suggests that the virus could still be in a dormant stage after germination. Alternatively, viral genome translation may be at a very low level, and does not generate sufficient GFP for visualization by confocal microscopy at this stage.

I thus considered choosing seeds with a high degree of infection for such study. Seeds with mottled seed coats, harvested from infected plants, are considered to be a good indicator of viral infection, but non-mottled seeds cannot be ruled out from virus infection. This is because it was previously shown that mottled and non-mottled seeds transmit virus nearly equally efficiently (Bos, 1972; Hill and Benner, 1980; Bajwa and Pacumbaba, 1996). Seed coat mottling or seed coat discoloration is sometimes induced by low temperature (Takahashi and Abe, 1994; Morrison *et al.*, 1998, Kasai *et al*., 2009). Other environmental factors such as dampness and high humidity, especially during storage, can greatly affect seed coat color and lower soybean seed quality. Soybean plants that are exposed to low temperatures (approximately 15 ℃) during flowering showed identical patterns of seed coat mottling that are also dependent upon the *I* locus and resulted from inhibition of RNA silencing at low temperatures (Kasai *et al*., 2009). However, virus-induced seed coat mottling is a common factor in infected plants. During seed development, soybean varieties that have the dominant *I* allele exhibited low chalcone synthase (CHS) mRNA activity when compared to the pigmented seeds (Wang *et al*., 1994). Post-transcriptional silencing of the chalcone synthase gene cluster by a viral suppressor protein has been proposed by Senda *et al.* (2004) and Tuteja *et al.* (2004)

as being responsible for pigmentation in the seed coat of black colored soybean seeds. However, a spontaneous mutation in the dominant *I* allele had a similar effect, and produced black colored soybean seeds (Lindstrom and Vodkin, 1991; Wang *et al*., 1994). The mottling of soybean seed coats in plants infected with SMV results from partial suppression of RNA silencing of the CHS mRNAs by SMV HC-Pro (Senda *et al*., 2004).

In the current study, both mottled and non-mottled seeds harvested from infected Williams 82 cultivar showed SMV transmission during seedling stage. As shown in Figure 26, mottled seed showed greater efficiency of SMV transmission (15% to 26%) when compared to non-mottled seed (0% to 14%). In nature, up to 30% or more of seed from infected soybean plants carry SMV, whereas seed transmission values between 0% and 64% were observed in 12 separate cultivars (Ross, 1970; Porto *et al.*, 1975; Kasai *et al*., 2009). The degree of seed transmission of SMV in the Williams 82 cultivar calculated from my experiments was in the range of typical SMV infection rates. However, regardless of mottled or non-mottled seeds used, I failed to find the exact timing and location of virus replication during any seedling stage. A close monitoring of seedling phenotypes from mottled seeds revealed that two weeks after germination, the first unifoliate leaf showed typical mosaic or chlorosis symptoms (Figure 25C). As expected, plants grown from healthy seeds did not exhibit any viral symptoms in the leaf during the seedling stage. Based on this observation, SMV must establish its infection within two weeks after germination. Future research should be directed to investigate when and where SMV re-initiates its replication in this two-week time period.

5 CONCLUSION

Seed-transmission of viruses means that the virus (usually located in the embryo) is capable of establishing replication from within naturally germinating seedlings. The presence of virus in the seed does not always lead to seedling infection. That is, SMV may be present in the seed, but still might not be transmitted parentally if it does not reach the embryo. Virus infection of embryos may be essential for virus seed transmission in most systems, although the presence of virus in the seed coat and endosperm tissue does play a role in the seed transmission for some viruses. In this study, I conclude that SMV infects all parts of the seed, including embryo, cotyledon and testa, and embryo infection is likely via the suspensor. My results pave the way for further analyses of SMV infection via seed transmission. For example, it would be interesting to understand why only some seeds become infected in SMV infected soybean plants. It also would be meaningful to determine when and where SMV reinitiates its replication within seed tissue after germination. A deeper insight into the mechanisms behind seed transmission and continual identification of seed transmission rates of different cultivars could instruct novel methods of control for SMV, thus increasing the profitability of soybean growers in Canada and the rest of the world.

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APPENDIX A: Copyright Clearance of Figure 2

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Number of charts/graphs/tables/figures	1
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APPENDIX B: Media Composition

MS Germination medium

Component	Amount/L
B5 major salt, 10X	2.165 g
B5 minor salt, 10X	100 mL
Ferrous-NaEDTA, 100X	10 mL
Sucrose, 2%	20 g
Adjust to pH to 5.8 with NaOH	
Adjust with ddH_2O to 1 Liter.	
Add Phytagel ™	3g
Autoclave 20 min at liquid cycle. Add	
B5 vitamin, 100X	10 mL
Mix well and pour 25 plates /L at 50 °C	

APPENDIX C: Composition of Buffers and Solutions

Boehringer Block solution 1

Boehringer Block reagent	1 g
TBS (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl)	to 200 mL

It takes one hour (or longer) to dissolve by heating it to 60-70 ℃

Block solution 2/ Buffer 2

Bovine Serum Albumin	10 g
Triton X-100	3 mL
TBS (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl)	to 1 liter

Buffer 3

For 1 liter	
5M NaCl	20 mL
1M Tris-HCl pH 9.8-9.9	100 mL
1M $MgCl_2$	50 mL
H_20	to 1 liter

pH-9.5 and prepared fresh in each time

Formamide

Add ~5g of ion exchange resin per 100 ml of formamide to the formamide. Stir ~30min RT. Filter through Whatman or Millipore (or decant). Store deionized formamide at –20 ℃.

FAA (37% Formaldehyde/Glacial Acetic Acid/Alcohol) (100 mL) – in hood

95% Ethanol	50 mL
37% Formaldehyde	10 mL
Glacial Acetic Acid	5 mL
DEPEC H_2O	35 mL

10% Glycine (Rnase-free)

10g glycine in 100 mL DEPEC water

Pre-Hybridization solution (Rnase-free)

For 8 ml	
Deionized Formamide	4 mL
50% Dextran sulphate	2 mL
10X Salts	1 mL
tRNA (100 mg/ml in DepC water)	100 µL
50X Denhardt's reagent	200 µL
DEPEC	700 µL

Heating helps the dextran sulphate to dissolve, Avoided putting in ice.

Note: Pre-hybridization solution considered as hybridization solution when probe is added to this prehybridization solution.

10X PBS (Rnase-free)

For 1 liter	
NaCl	74 g
Na_2HPO_4	9.94 g
NaH_2PO_4	4.14 g
Millipore water	to 1 liter

Add 1 mL DEPC, stir overnight and autoclave to inactivate DEPEC.

10X Salts (Rnase-free)

For 50 mL	
5M NaCl (in DEPEC H_2O)	30 mL
1M Tris-HCl pH 6.5 (in DEPEC H_2O)	5 mL
0.5M EDTA (in DEPEC H_2O)	5 mL
$NaH_2PO_4.H_2O$	3.9 g
Na_2HPO_4 (anhydrous)	3.55 g
DEPEC Water	to 50 mL

5M NaCl (Rnase-free)

NaCl	292.2 g
Millipore water	to 1 liter

Add 1 ml DEPEC, stir overnight, autoclave to inactivate the DEPEC.

20X SSC (Rnase-free)

For 1 liter	
NaCl	175.3 g
Sodium citrate	88.2 g
Millipore water	to ~ 900 mL

Brought to pH 7 with HCl, then to 1 liter with Millipore water. 1 mL DEPEC added and stirred overnight. Autoclaved to inactivate DEPEC.

TBS

For 1 liter	
1M Tris pH 7.5	100 mL
5M NaCl	20 mL

Millipore water to 1 litre

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Poulin-Laprade, D., Bashar, T. & Burrus, V. 2013.Characterization of SetCD-binding sites in the integrating conjugative elements of the SXT/R391 family and the genomic islands they mobilize. ASM 2013, Denver, Colorado, USA.

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