VIRUS VECTOR GENE INSERTS ARE STABILIZED IN THE PRESENCE

OF THE SATELLITE PANICUM MOSAIC VIRUS COAT PROTEIN

A Thesis

by

ANTHANY LAURENCE EVERETT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2008

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,   Karen-Beth G. Scholthof
Committee Members,   Herman B. Scholthof
                           Julian Leibowitz
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ABSTRACT

Virus Vector Gene Inserts Are Stabilized in the Presence of

the Satellite Panicum Mosaic Virus Coat Protein. (August 2008)

Anthany Laurence Everett, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Karen-Beth G. Scholthof

The coat protein of satellite panicum mosaic virus (SPMV) was used to stabilize viral vector gene inserts *in planta*. A *Potato virus X* (PVX) vector carrying the SPMV capsid protein (CP) gene was successfully stabilized through three serial passages in *Nicotiana benthamiana* from the upper non-inoculated leaves following rub inoculation. The presence of SPMV CP expression from the PVX vector was confirmed by necrotic lesions that occur only when SPMV CP is present and by western blot and reverse-transcription PCR analyses. In addition, PVX-SPCP was co-inoculated onto *N. benthamiana* with a *Tomato bushy stunt virus* vector carrying a green fluorescent protein gene, which normally does not yield GFP expression in upper tissue due to loss of the insert. However, upon co-inoculation with PVX-SPCP, upper non-inoculated leaves exhibited GFP accumulation based on green fluorescence by UV illumination at 488 nm and western blot analysis. GFP expression was more abundant in upper non-inoculated *N. benthamiana* leaves as well as systemic tissues when the co-inoculation experiments were performed at 20°C compared to 25°C. These results suggest that SPMV CP is a viable molecular tool for stabilizing viral vector gene inserts *in planta*.

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Finally, thanks to my mother, brother, and sisters, for their encouragement and to Gabriel Faith Kuty for her patience and love.

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CHAPTER I

INTRODUCTION

St. Augustine Decline Disease

St. Augustinegrass (*Stenotaphrum secundatum*) is a dark-green, broad-leaf, turf-grass that is grown in tropical and sub-tropical areas of the world including Mexico, Australia, Africa, the Caribbean and Hawaiian Islands, and the United States Gulf Coast region. A disease of St. Augustinegrass, St. Augustine Decline (SAD) Disease, was first described in 1969 in the United States (McCoy, Toler, and Amador, 1969), and has subsequently been reported in almost all the Gulf Coast region states (Cabrera and Scholthof, 1999). SAD, which causes millions of dollars in St. Augustinegrass damage annually, is characterized by turf thinning and browning. The disease is spread mechanically, most often by landscaping practices such as mowing and trimming. To date, no known insect vectors exist. In individual plots or lawns, SAD develops over the course of several years and severity ranges from trace to complete turf loss. Individual plants show a characteristic chlorotic mottle or mosaic that runs parallel to the mid-rib of the leaf (Buzen et al., 1984; Cabrera and Scholthof, 1999) (Fig. 1).

Panicum mosaic virus **and satellite panicum mosaic virus**

Panicum mosaic virus (PMV) is the causal agent of St. Augustine Decline Disease (SAD) (Cabrera and Scholthof, 1999; McCoy, Toler, and Amador, 1969). PMV

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This thesis follows *Virology* format.

FIG. 1. Symptoms of St. Augustine Decline Disease on St. Augustinegrass. Healthy (left) and symptomatic (right) leaves of St. Augustinegrass. The yellow mosaic or mottle occurs during infection of PMV or a mixed infection of PMV plus SPMV (Cabrera and Scholthof, 1999). Samples were harvested in April, 2008 from a private residence in College Station, TX.

was first reported in switchgrass (*Panicum virgatum*) (Sill and Pickett, 1957). An infection of St. Augustinegrass with PMV results in the typical symptoms described previously, and PMV virions readily accumulate in the wild, the greenhouse, or under controlled conditions in the laboratory (Holcomb, Liu, and Derrick, 1989).

PMV is the type species of the genus Panicovirus, in the family Tombusviridae (Turina et al., 1998). PMV is a 4,326 nucleotide (nt) positive-sense single-stranded RNA (ssRNA) genome that encodes six proteins (Turina, Desvoyes, and Scholthof, 2000; Turina et al., 1998) (Fig. 3). A 48 kDa and a 112 kDa protein are translated directly from the 5'-end of the genome following the P48 ORF (Batten, Turina, and Scholthof, 2006; Turina et al., 1998). The 112 kDa protein is expressed by read-through of an amber stop codon (UAG) (Batten, Turina, and Scholthof, 2006; Turina et al., 1998). P48 and P112 form the replicase complex responsible for minus-strand and sub-genomic RNA (sgRNA) synthesis and viral genome replication (Batten, Turina, and Scholthof, 2006). The four remaining genes are translated from a single sgRNA. The sgRNA encodes P8 and P6.6 which are involved in cell-to cell movement (Turina, Desvoyes, and Scholthof, 2000). P6.6 is produced by leaky scanning of the start codon of P8. The capsid protein (P26), encoded downstream of P6.6, may be expressed from a yet unidentified internal ribosomal entry site (IRES) (Turina, Desvoyes, and Scholthof, 2000; Turina et al., 1998). To date, the only known function of PMV CP is encapsidation of PMV and SPMV gRNA (Qiu and Scholthof, 2000). The P15 ORF is nested in the CP gene, expressed by leaky scanning, and helps in systemic movement (Turina et al., 1998).
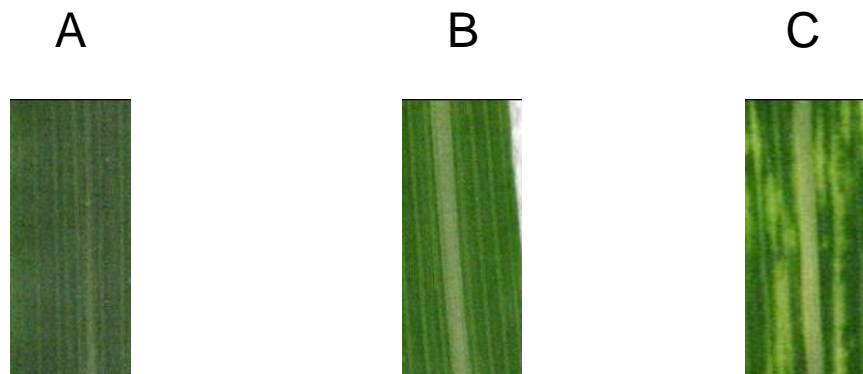
FIG. 2. Symptoms associated with a PMV alone or PMV plus SPMV infection on proso millet (*Panicum miliaceum*) plants. A) Healthy proso millet cv. Sun-up. B) An infection of proso millet by PMV alone results in mild mosaic symptoms and C) co-infection with PMV plus SPMV results in severe symptom development. Upper non-inoculated/systemic leaves of proso millet cv. Sun-up were photographed at 14 days post inoculation.
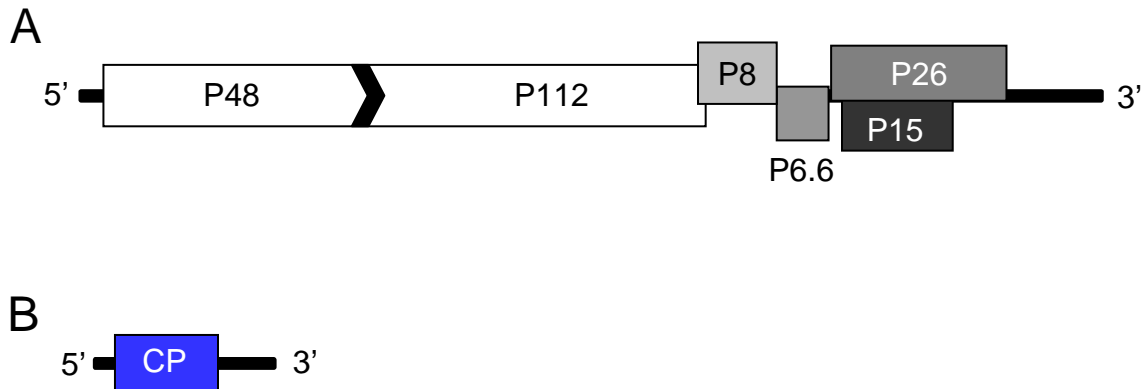
FIG. 3. The genomes of PMV and SPMV. A) The 4,326 nucleotide (nt) genome of *Panicum mosaic virus*. P48 and P112 form the replicase complex, and P112 results from read-through of an amber (UAG) stop codon (black chevron). The rest of the viral proteins (small colored bars) are expressed from a sub-genomic RNA (lower line). P8 and P6 are associated with cell-to-cell movement. P15 contributes to systemic movement and is expressed because of a leaky start codon at the 5'-end of the 26 kDa CP (P26) ORF. B) The 824 nt genome of satellite panicum mosaic virus. The SPMV genome carries a 438 nt CP gene (blue box). The black lines represent untranslated regions.

smaller, 16 nm particle (Buzen et al., 1984). These smaller particles were classified as satellite panicum mosaic virus (SPMV) (Buzen et al., 1984; Cabrera and Scholthof, 1999), and were shown to be inconsequential to symptom development in St. Augustinegrass (Cabrera and Scholthof, 1999; Holcomb, Liu, and Derrick, 1989). Contrarily, SPMV induces a synergism of symptom development in proso millet (*Panicum miliaceum* L.) and foxtail millet (*Setaria italica* L.) plants during a mixedly infection with PMV (Scholthof, 1999b) (Fig. 2).

SPMV is an 824 nt positive-sense ssRNA that expresses a 17 kDa capsid protein (Masuta et al., 1987; Turina et al., 1998) (Fig. 3). The X-ray crystalline structure of the SPMV virion (Ban and McPherson, 1995) show that sixty subunits of the jelly-roll motif capsid protein interconnect to form an icosahedral lattice with three subunits per face of the icosahedron (T=1) (Ban and McPherson, 1995; Makino, Larson, and McPherson, 2005).

SPMV requires PMV, its helper virus, for replication and movement, in host plants (Buzen et al., 1984; Turina et al., 1998), but has minimal sequence homology with the PMV genome, sharing only 7 nt at the 5'-end and 3 nt at the 3' end, presumably used for recognition by the PMV replicase complex. Infectious clones of PMV and SPMV allow for manipulation of the cDNA of the viral genomes (Masuta et al., 1987; Turina et al., 1998). The cDNAs are transcribed *in vitro* and the resultant RNA is rubbed on a suitable host to produce an infection (Cabrera and Scholthof, 1999; Turina et al., 1998).

To understand the biological roles of the proteins expressed by PMV and SPMV a reverse genetic approach using infectious cDNA has been employed (Batten et al.,

2006; Batten, Turina, and Scholthof, 2006; Desvoyes and Scholthof, 2000; Omarov, Qi, and Scholthof, 2005; Qi, Omarov, and Scholthof, 2008; Qi and Scholthof, 2008; Qiu and Scholthof, 2000; Qiu and Scholthof, 2004; Scholthof, 1999b). The N- and C-terminus of SPMV CP were shown to have multiple biological functions, including, but not limited to, RNA binding, helper virus interactions, symptom development and systemic movement. From this the basic functions of the proteins have been determined (Batten et al., 2006; Batten, Turina, and Scholthof, 2006; Omarov, Qi, and Scholthof, 2005; Qi, Omarov, and Scholthof, 2008; Qi and Scholthof, 2008). As an additional tool to elucidate protein function, SPMV CP has also been expressed from two plant virus gene vectors. Using the viral vector expression systems, SPMV CP was able to induce symptoms on a non-host and interfere with a suppressor of gene silencing, P25, from *Potato virus X* (PVX) (Qiu and Scholthof, 2004).

Plant virus gene vectors

Several plant viruses have been genetically modified to be used as virus gene vectors. These viruses can be used to express a foreign gene or RNA in host plants. This strategy was developed because plant virus vector expression of foreign genes provides higher concentrations and more rapid results when compared to more traditional methods such as transgenic expression (Canizares, Nicholson, and Lomonossoff, 2005; Gleba, Klimyuk, and Marillonnet, 2007; Scholthof, Mirkov, and Scholthof, 2002). The major limitation to using viral vector expression systems has been the instability of the
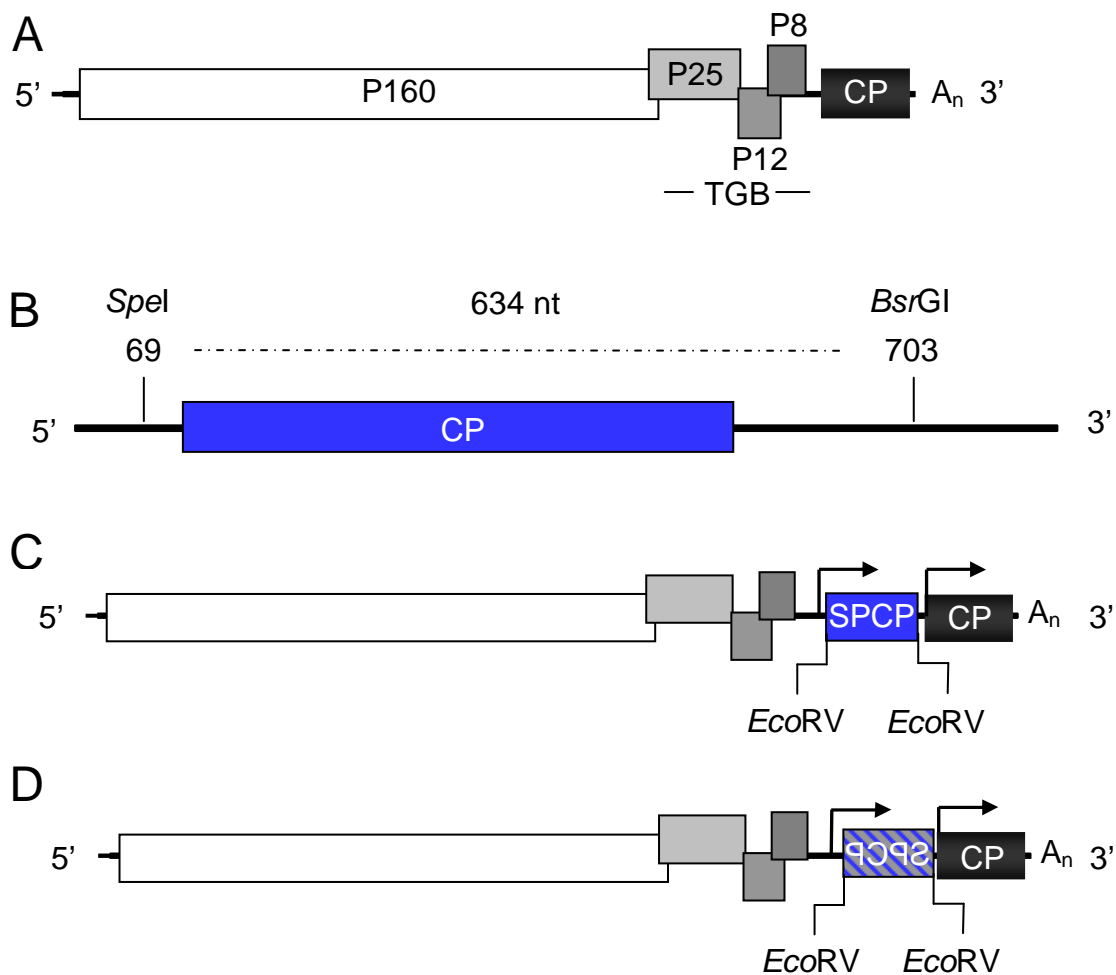
FIG. 4. *Potato virus X* and the *Potato virus X* vectors carrying the satellite panicum mosaic virus coat protein gene. A) The 6,435 nt genome of *Potato virus X* (PVX) has a 5'-cap (thin black line) and a 3' poly(A) tail (Huisman et al., 1988; Skryabin et al., 1988). The thick black bar represents untranslated regions. The P160 protein forms the viral replicase. The P25, P12, and P8 proteins form the triple gene block (TGB) and are responsible for suppression of gene silencing, systemic and cell-to-cell movement, respectively. P25 is produced from sgRNA1. P12 and P8 are produced from sgRNA2, and the coat protein (CP) is produced from sgRNA3. B) The full-length infectious cDNA of satellite panicum mosaic virus (SPMV) was cleaved at the *Spe*I and *Bsr*GI restriction enzyme sites and blunt-ended using Klenow polymerase, resulting in a 634 bp fragment encoding the SPMV CP. C) PVX vectors PVX-SPCP+ and D) PVX-SPCP- were made by inserting the 634 nt fragment containing the SPMV CP gene in a coding and non-coding orientation at the *Eco*RV restriction enzyme site downstream of a duplicated PVX CP promoter, respectively (bent arrow). Note that the figures are not to scale.
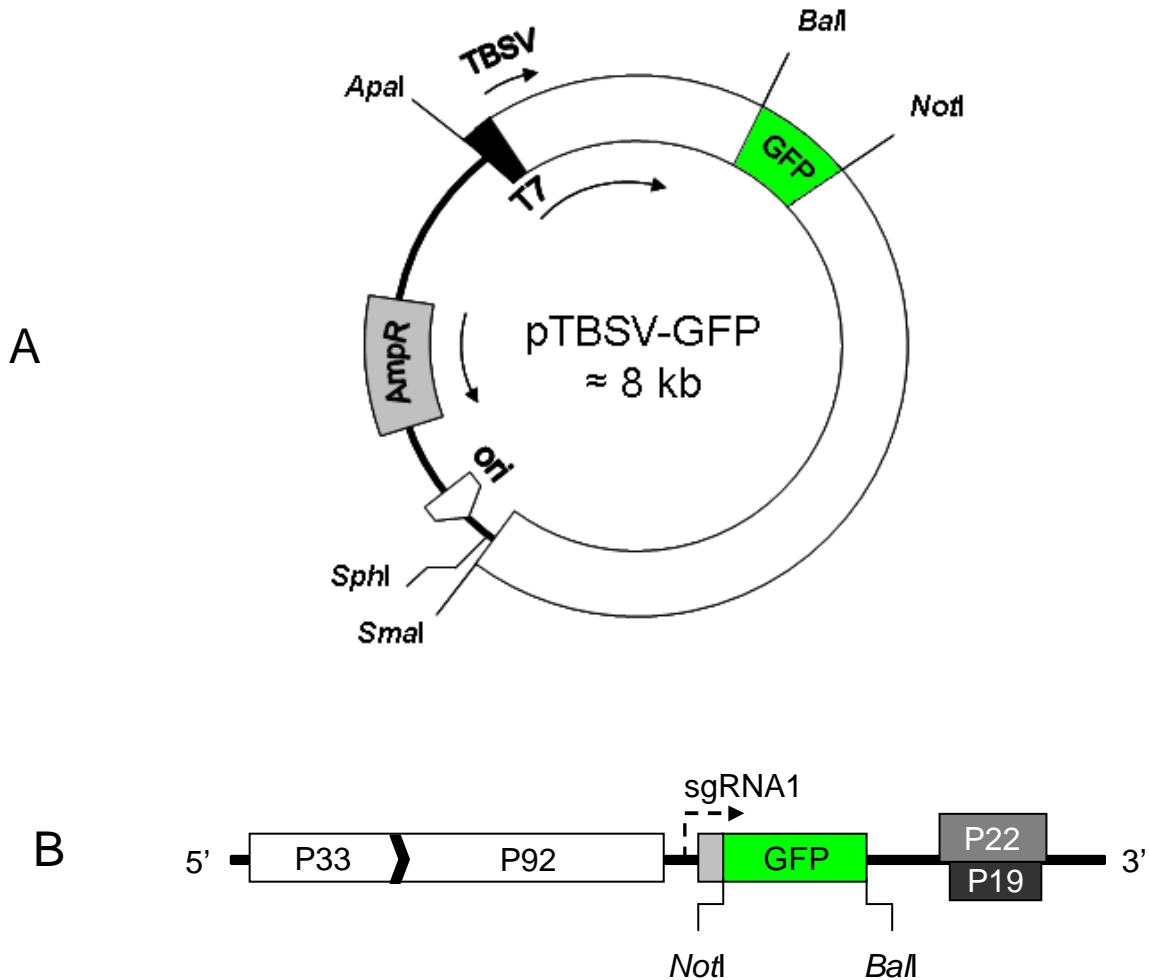
FIG. 5. Maps of pTBSV-GFP and TBSV-GFP. A) A circular map of pTBSV-GFP. The plasmid is a modified pUC119 with an origin of replication (ori), a β-lactamase gene (AmpR) (medium curved arrow), and a T7 polymerase promoter (large curved arrow). Restriction enzyme sites *Apa*I, *Sph*I, and *Sma*I are marked. A GFP insert (green box) is located between the *Not*I and *Bal*I sites of TBSV cDNA. The 5'-end of TBSV cDNA is marked (short curved arrow). Drawing not to scale. B) A linear map of the ca. 4,800 nt genome of a full-length infectious clone of TBSV carrying the ca. 700 nt GFP gene. Translation from sgRNA1 (broken arrow) results in a CP:GFP fusion protein that consists of the 17 N-terminal residues (light grey box) of the TBSV CP, and GFP (green box). The remainder of the TBSV CP gene is out of frame and downstream of the *Bal*I site.

gene insert and subsequent loss of foreign gene expression (Avesani et al., 2007; Gleba, Klimyuk, and Marillonnet, 2007; Scholthof, Mirkov, and Scholthof, 2002).

In this study, we used two plant virus vectors, *Potato virus X* (PVX) (Figs. 4A, C-D) and *Tomato bushy stunt virus* (TBSV) (Fig. 5), to express SPMV CP and the green fluorescent protein (GFP) gene. A PVX vector, PVX-E1S, was modified to abolish a *Sal*I restriction enzyme site and to use a duplicated coat protein promoter for independent gene expression from a sgRNA (Fig. 4) (Chapman, Kavanagh, and Baulcombe, 1992; Qiu and Scholthof, 2004). A TBSV vector expresses an enhanced green fluorescence protein (eGFP) that has been optimized with plant codons to recruit plant derived t-RNA, resulting in increased GFP fluorescence *in planta* (Fig. 5).

Expression of foreign proteins from either the PVX vector or the TBSV vector is eventually lost during plant virus gene vector infection. PVX has been shown to lose segments of the gene insert, and thus expression, both in initially and serially passaged plants, and additionally, insert instability increases with insert size (Avesani et al., 2003; Avesani et al., 2007; Chapman, Kavanagh, and Baulcombe, 1992; Qiu and Scholthof, 2004). TBSV vectors have been shown to lose gene expression before reaching systemic tissues of the host plant (Borja et al., 1999; Scholthof, 1999a; Scholthof, Morris, and Jackson, 1993). The loss of gene insert expression from plant virus gene vectors makes them ideal tools to facilitate stabilization experiments, for if foreign gene expression was not lost from these vectors, stabilization studies would be moot.

SPMV and its coat protein have multiple functions

We have observed that a SPMV mutant that did not express CP generated increased numbers of defective interfering RNAs (DIs) (Qi and Scholthof, 2008; Qiu and Scholthof, 2001a; Qiu and Scholthof, 2001b). In addition the concentration of its helper virus, PMV, was increased *in planta* when co-infected with SPMV (Qiu and Scholthof, 2001b; Scholthof, 1999b). The increase in PMV titer and the decrease in SPMV DI formation suggested that SPMV CP may play a crucial role in maintaining the integrity of both viral genomes. During another study investigating the role of SPMV in virus induced gene silencing, GFP expression from TBSV-GFP (Fig. 5) was occasionally observed in upper non-inoculated leaf tissues of *N. benthamiana* during a co-infection with a PVX vector expressing the satellite panicum mosaic virus coat protein gene (PVX-SPCP+) (Qiu and Scholthof, 2004). When *N. benthamiana* was inoculated with TBSV-GFP alone, GFP expression was only observed in the inoculated leaves (Qiu and Scholthof, 2004).

My hypothesis, taking the previous results together, is that SPMV CP may be able to be used as a molecular tool to maintain vector genome integrity or gene insert stability. In this study I investigated if SPMV can fulfill such a role in *N. benthamiana*. For this, serial passages of PVX-SPCP+ was used to test the viability of this hypothesis of *cis*-stabilization of a foreign gene. Similarly, co-infection with TBSV-GFP plus PVX-SPCP+ was used to test for *in trans*-stabilization of a foreign gene insert (GFP).

CHAPTER II

MATERIALS AND METHODS

Plant stocks

Approximately 50 *N. benthamiana* seeds were spread on Metro Mix soil (SunGro Horticultural Distribution, Bellevue, WA) in a 4 in pot and placed in a growth chamber (25°C, 14 h dark and 10 h light) with regular watering. Three to four days after cotyledon emergence, individual seedlings were transplanted to 4 in pots filled with Metro Mix soil and maintained at 25°C, 14 h dark and 10 h light prior to inoculation at the 4-6 leaf stage.

Preparation of infectious RNA stock

The insertion of the SPMV CP gene into the PVX vector was previously described (Qiu and Scholthof, 2004). Briefly, a full-length infectious SPMV cDNA (pSPMV-1) (Turina et al., 1998) was digested with *Spe*I and *Bsr*GI at positions 69 and 703, respectively (Fig. 4B). Following treatment with Klenow polymerase (Large fragment), this resulted in a 634 bp fragment (SPCP) which includes the complete 473 nt SPMV CP gene flanked by 19 and 142 bases on the 5'- and 3'-ends, respectively. SPCP was ligated in a coding and reverse orientation using ligase at 4°C overnight into a modified pPC2S plasmid at the *Eco*RV site (nt 5677) (Qiu and Scholthof, 2004). The modification abolished the *Sal*I restriction enzyme site at position 6572 to form PVX-

E1S cDNA (Qiu and Scholthof, 2004) from the original pPC2S plasmid (Chapman, Kavanagh, and Baulcombe, 1992).

The ligation mix containing approximately 7.5 µg of plasmid DNA was added to 100 µL of *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA) competent cells and was heat shocked for 3 min at 42°C. The transformed cells were plated on Luria-Bertani (LB) agar with 100 mg ampicillin/L (Sigma, St. Louis, MO) media that was pre-warmed in a 37°C incubator for 15 min and incubated overnight at 37°C. Single colonies were selected and grown overnight at 37°C in 2 mL of LB liquid media with 100 mg ampicillin/L media (LB-Amp). The 2 mL culture was added to 48 mL of LB-Amp and grown over-night at 37°C. Plasmid DNA was isolated with a Qiagen Maxi-prep kit (Qiagen, Valencia, CA), linearized with *Spe*I, and transcribed using T7 polymerase, as described previously (Qiu and Scholthof, 2004; Scholthof, Morris, and Jackson, 1993). The integrity of the transcripts was confirmed by loading 3 µL of transcription solution mixed with 1 µL loading dye (50% glycerol, 1 mM EDTA pH. 8.0, 0.4% xylene cyanol, 0.3% bromophenol blue) onto a 1% agarose gel and electrophoresed at 120 V for 45 min in 1X TBE buffer (8.9 mM Tris, 88.9 mM sodium borate, 4 mL 0.5 M EDTA, pH 8.0). RNA bands were visualized by soaking the agarose gels in 500 mL of TBE buffer containing 1 drop of 1 M ethidium bromide for 15 min followed by UV illumination at 365 nm.

TBSV-GFP was derived from the plasmid pTBSV-GFP containing TBSV with an enhanced GFP insert, a kind gift from Teresa Rubio and A. O. Jackson (Univ. of California, Berkeley, CA). Briefly, a GFP gene constructed to use plant optimized

codons (eGFP) was ligated between the *Not*I and *Bal*I restriction enzyme sites of the pTBSV-100 plasmid carrying an infectious cDNA clone of TBSV downstream of a T7 promoter (Hearne et al., 1990) (Fig. 5A). This fused the eGFP coding sequence approximately 50 nt downstream and in frame with the TBSV CP gene start codon resulting in a ca. 30 kDa N-terminal CP:eGFP protein during translation (Yamamura and Scholthof, 2005) (Fig. 5). PTBSV-GFP was isolated as described above and linearized with *Sma*I (Fig. 5A). *In vitro* transcripts of TBSV-GFP (Fig. 5B) were rub-inoculated to *N. benthamiana* using routine protocols (Scholthof et al., 1995).

Passage of PVX-derived constructs in *Nicotiana benthamiana* plants

RNA inoculation mixtures were prepared by adding 10 µL of PVX-SPCP+, PVX-SPCP-, or PVX-E1S transcripts to 90 µL of RNA inoculation buffer (0.05 M K_2HPO_4, 0.05 M glycine, 1% bentonite, 1% Celite, pH 9.0), as previously described (Qiu and Scholthof, 2004). Two-week old *N. benthamiana* plants were rub-inoculated with 10 µL of RNA inoculation mix or RNA inoculation buffer alone onto each of the 4th, 5th, and 6th true leaves (Fig. 6A). Inoculated plants were transferred to a cardboard box, covered with moistened BenchGuard paper (International Product Supplies, London, England), and kept at room temperature overnight. The following morning, plants were removed to a 25°C growth chamber with 10 h light and 14 h dark.
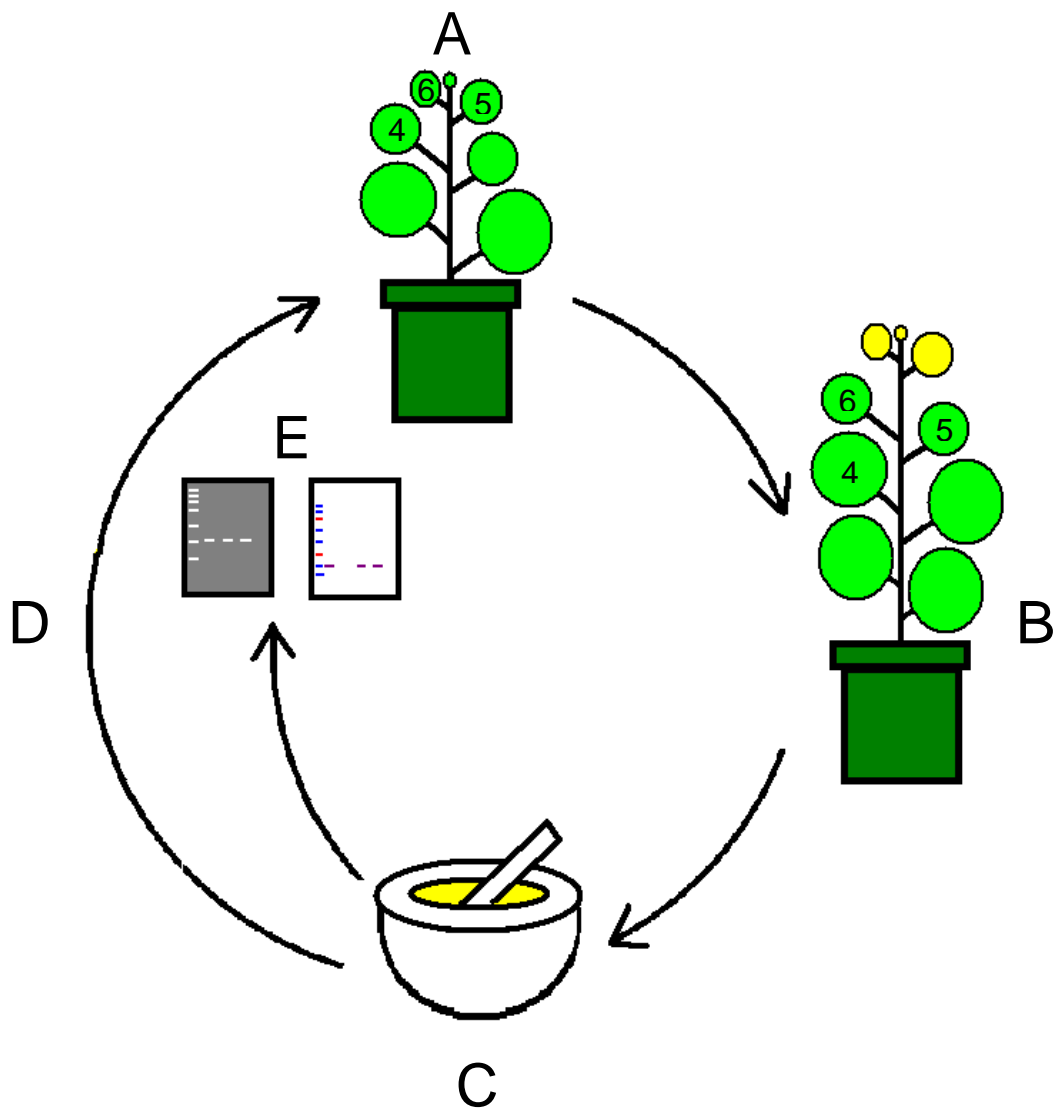
FIG. 6. Passage of PVX constructs in *N. benthamiana*. A) The 4th, 5th, and 6th leaves of two-week old *N. benthamiana* plants were inoculated with inoculation buffer, PVX-E1S, PVX-SPCP+, or PVX-SPCP-. B) The inoculated plants were inspected for necrotic lesions in upper non-inoculated tissues at 11-14 days post inoculation (yellow). C) The symptomatic upper tissue was ground in a mortar with a pestle and the extract was used for subsequent passages and molecular analyses. D) Homogenized tissue from symptomatic upper leaves (as in B) was rub inoculated (passaged) onto healthy two-week old *N. benthamiana* plants or E) used for RT-PCR and western blot analyses.

If PVX-SPCP+ plants were symptomatic at 11-14 dpi (Fig. 6B), then 0.5 g of uppermost non-inoculated symptomatic leaf tissue was harvested and ground in a mortar with a pestle in 1 mL virus inoculation buffer (0.05 M K_2HPO_4 pH 7.4, 1% celite) (Fig. 6C) and mechanically passaged onto the 4th 5th, and 6th leaves of two-week old healthy *N. benthamiana* plants (Fig. 6D). The passages were repeated until i) SPMV-CP indicative local lesions were no longer apparent by direct observation in upper non-inoculated plant tissues, ii) no protein was present in upper leaf tissues as determined by western blot, iii) and no SPMV CP RNA was present as determined by reverse transcription (RT) PCR (Fig. 6E). Three independent studies with five replicates per study were analyzed.

Co-infection of TBSV-GFP with PVX-SPCP in *Nicotiana benthamiana*

RNA inoculation mix was prepared by adding 10 µL TBSV-GFP and PVX-SPCP+ transcripts each to 80 µL of RNA inoculation buffer. Plant inoculations, passages, and maintenance were described above. Upper non-inoculated leaves were photographed and harvested at 7-9 dpi. Three independent experiments were conducted using five replicates at 20°C. Three independent experiments were also performed using three replicates for each experiment at 25°C.

Immunoblot analyses

Western blot analyses of upper non-inoculated leaf tissues was performed by harvesting 0.5 g symptomatic leaf tissue, grinding it in an ice-cold mortar with a pestle

with 1 mL 2X STE buffer (0.1 M Tris, 0.01 M EDTA, 2% SDS, pH 8.0). Homogenized

tissue was transferred to a 1.5 mL micro-centrifuge tube before one-fifth volume of 5X

cracking buffer (3.8 g Tris, 5 g SDS, 5 mL β-mercaptoethanol, 5 mg bromophenol blue,

50 mL glycerol, 45 mL dH$_2$O, pH. 6.8) was added. The sample was then boiled for 15

min. A 40 µL aliquot was electrophoresed through an SDS-polyacrylamide gel (SDS-

PAGE) (Running gel: 15% acrylamide, 1% SDS, pH 8.8; Stacking gel: 0.05%

acrylamide, 1% SDS, pH 6.8) in Laemmli running buffer (0.5 M Tris, 1.9 M glycine, 1%

SDS) (Laemmli, 1970) for 1.5 h at 120 V. Proteins were electro-transferred from the

SDS-polyacrylamide gel to a nitrocellulose membrane (Pall, New York, New York) in

transfer buffer (0.025 M Tris, 0.25 M glycine, 20% methanol) for 90 min at 265 mA

(Bio-Rad electro-transfer apparatus, Bio-Rad Laboratories, Hercules, CA). Blots were

soaked in 7.5% non-fat dried milk (Carnation, Solon, OH) in TBS/Tween (0.5 M Tris,

0.2 M NaCl, 0.05% Tween-20) at 4°C overnight using standard protocols (Sambrook,

1989). The blot was washed 3 times for 15 min each in TBS/Tween prior to adding 10

mL of antiserum containing polyclonal rabbit antibodies raised against SPCP or mouse

antibodies raised against GFP or TBSV P19 (1:10,000 dilution in 10 mL 7.5% milk-

TBS/Tween buffer). The blot was incubated with antibody serum at room temperature

with gentle rotation for 4 h. The membrane was again washed 3 times in TBS/Tween for

15 min followed by serum containing goat anti-rabbit or goat anti-mouse IgG antibodies

conjugated to alkaline phosphatase (1:2,000 dilution in 10 mL 7.5% milk-TBS/Tween

buffer, Sigma, St. Louis, MO) at room temperature for 4 h with gentle rotation. After

washing in TBS/Tween again as above, the protein bands were then visualized by the

addition of 33 µL 5-bromo-4-chloro-3 indolyl phosphate p-toluidine (Sigma, St. Louis, MO), 66 µL nitrotetrazolium blue salt (Sigma, St. Louis, MO) in 1X alkaline phosphatase buffer, pH 9.5 for 3-5 min.

RNA analyses

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of PVX-SPCP from passaged plants was carried out on RNA isolated from 0.5 g of upper non-inoculated *N. benthamiana* leaves. Harvested tissue was ground in an ice-cold mortar with a pestle in 500 µL 2X STE buffer followed by the addition of 500 µL 1:1 phenol-chloroform (pH 7.4). The homogenate was transferred to a 1.5 mL micro-centrifuge tube and extracted 3 times by centrifugation at 10 K rpm for 10 min at 4°C, taking the aqueous layer each time and adding it to 500 µL 1:1 phenol-chloroform, and mixed by inversion after each centrifugation. The aqueous layer was then transferred to a clean 1.5 mL micro-centrifuge tube followed by addition of an equal volume of 100% ethanol and 1:10 (v/v) 3 M sodium acetate (pH 5.2) and placed at -65°C overnight. The RNA was pelleted by centrifugation at 10 K rpm for 10 min at 4°C. The supernatant was decanted. The pellet was washed with ice-cold 70% ethanol and re-centrifuged at 10 K rpm for 10 min at 4°C. After decanting the supernatant again, residual ethanol was removed from the pellet by vacuum centrifugation (DNA Speed Vac, Savant, Waltham, MA). The pellet was re-suspended in 40 µL dH$_2$O. A forward primer (5'-**GCATCGATCG**CTAGTCTCACG-3') and reverse primer (5'-**TCGACGATAT**CACACGGTCGCC-3') pair were designed so that the ten 5'-

proximal nucleotides of each primer corresponded to the flanking 10 nt of the PVX genome (bold). The eleven 3'-proximal nucleotides of the forward primer and the twelve 3'-proximal nucleotides of the reverse primer are complementary to the 5'- and 3'- end of the SPMV CP gene, respectively (Fig. 8). For, the RT-PCR, 1 ng each of the forward and reverse primers, and a 12-18 nucleotide oligo-dT primer (Invitrogen, Carlsbad, CA) were added to a pre-made RT-PCR bead (Ready-to-Go RT-PCR, GE Healthcare Bio-Sciences, Piscataway, NJ) plus 1 µL of RNA extracted from plant tissues and 46 µL of dH_2O. The amplification was carried out in an Applied Biosystems 2720 thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: 42°C, 30 min for first strand synthesis; 95°C for 5 min, to denature the cDNA; and 42 cycles at 95°C for 1 min, 55°C for 1 min; 72°C for 2 min. The cycle was ended after 72°C for 10 min, and then held at 4°C. The RT-PCR products were analyzed for the presence of the SPMV CP gene by gel electrophoresis using 15 µL RT-PCR product plus 3 µL loading dye on a 1% agarose gel for 45 minutes at 120 V in Tris-borate-EDTA (TBE) buffer (0.1 M Tris, 1 mM EDTA, pH 8.0). The DNA bands were visualized by exposing the gel to ethidium bromide in TBE buffer for 20 min before UV illumination at 395 nm.

Photography of plants

All photographs of *N. benthamiana* plants were taken with a Canon Powershot A630 (Canon, Lake Success, New York) using automatic ISO and focus settings. Picture size (resolution) was 3264 x 2448 pixels, and flash was used as needed. Photographs were manipulated by increasing/decreasing brightness and contrast or

cropping as needed. Nero Photosnap picture editing software was used for adjustments, and all pictures in individual figures were adjusted together. The aperture priority setting was used for pictures taken under a UV mercury lamp (H44GS100, Osram Sylvania, Danvers, MA) at 488 nm and ISO and focus was automatically controlled by the camera.

CHAPTER III

RESULTS

For this study, a multi-faceted approach was used to determine if SPMV CP expressed from a PVX virus gene vector can stabilize virus vector gene inserts during systemic infection *in planta*. Based on observational, biochemical, and molecular data, SPMV CP stabilization was shown to occur, by an as yet unknown mechanism, *in cis* and *in trans* using PVX- and TBSV- based vectors.

Cis-stabilization of the SPMV CP gene within a PVX vector by SPMV CP through serial passages in *Nicotiana benthamiana*

An infection of *N. benthamiana* with a PVX vector carrying a 634 nt insert from SPMV containing the 473 nt CP gene transcribed from a duplicated coat protein promoter (PVX-SPCP+) (Fig. 4) resulted in stunting and necrotic lesions along the Type II and Type III veins of the leaves (Fig. 7). In contrast, a PVX vector with the same SPMV 634 nt fragment in a reverse orientation (PVX-SPCP-) (Fig. 4) produced symptoms as PVX wild-type (Fig. 7). This was in agreement with previous observations (Qiu and Scholthof, 2004). For the preliminary study, the indicative symptoms of PVX-SPCP+ infected plants were used to test for SPMV CP expression through serial passages of PVX-SPCP constructs in *N. benthamiana*. At 25°C, upper non-inoculated leaves displayed necrotic lesions through at least three serial passages and in as many as five serial passages (Fig. 7, Table 1). This observation suggested that SPCP was stably

expressed, an atypical result, when compared to a plethora of other virus-gene vector reports (Avesani et al., 2007; Chapman, Kavanagh, and Baulcombe, 1992; Qiu and Scholthof, 2004; Scholthof, 1999a; Scholthof, Scholthof, and Jackson, 1996; Scholthof, Mirkov, and Scholthof, 2002).

To confirm that the necrotic lesions were specifically associated with the expression of SPMV CP during passage experiments, both western and RT-PCR analyses were conducted (Figs. 8-9). *N. benthamiana* plants were inoculated with PVX-SPCP+, PVX-SPCP-, or inoculation buffer to test for SPMV CP accumulation or as controls, respectively. The accumulation of SPMV CP was assayed with a polyclonal rabbit antiserum specific for SPMV CP. Immunoblots showed the presence of the SPMV CP 17 kDa monomer and a 34 kDa dimer product in both upper and lower non-inoculated leaves, although SPMV CP accumulation in lower systemic leaves is variable (Fig. 8). The latter result is most likely due to temporal and/or environmental effects during viral movement from source to sink tissues during virus infection. From this, SPMV CP accumulation as determined by western blot and necrotic lesions in upper non-inoculated tissues, leaves were selected to inoculate (passage) healthy, young plants. Both SPMV CP and the lesions were observed in the initially inoculated plants (passage 0) and continued through at least three and as many as five serial passages for only those plants inoculated with PVX-SPCP+ (Fig. 8, Table 1) In contrast, plants inoculated with PVX-SPCP- or inoculation buffer, as controls, had no detectable SPMV CP present, as expected, based on western blot, nor necrotic lesions in the initially inoculated plant or

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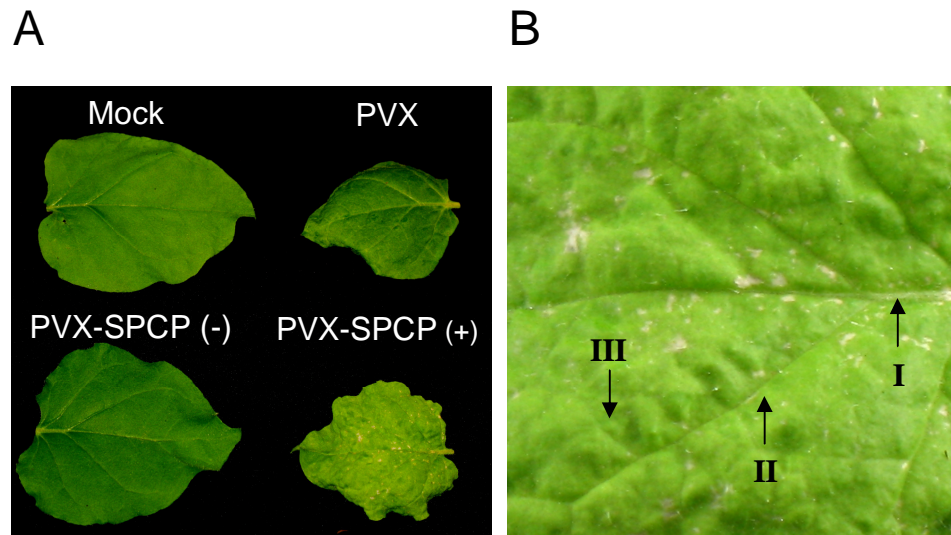
FIG. 7. Symptoms due to PVX-derivative infection on upper non-inoculated leaves of *N. benthamiana* plants at 14 days post inoculation. A) Representative upper non-inoculated leaves of *N. benthamiana* plants that were previously inoculated with inoculation buffer (Mock), infectious transcripts of PVX-E1S empty vector (PVX), or a PVX vector carrying the SPMV CP gene in an antisense (PVX-SPCP-) or coding (PVX-SPCP+) orientation 14 days before being removed. B) An enlargement of the PVX-SPCP+ leaf in (A). Necrotic lesions appear near Type I, Type II and Type III veins. Upper leaves were chosen for photographs.

TABLE 1

The number of *N. benthamiana* plants showing necrotic lesions at 11-14 days post inoculation (dpi) in upper non-inoculated leaves following serial passages.

Passage[b]	Inoculum[a]			
	Mock[c]	PVX[c]	SPCP-[c]	SPCP+[c]
0	0	0	0	13
1	0	0	0	13
2	0	0	0	11
3	0	0	0	10
4	0	0	0	3
5	0	0	0	1

a. *N. benthamiana* plants were inoculated with: inoculation buffer (Mock), PVX-E1S empty vector (PVX), PVX-SPCP- (SPCP-), or PVX-SPCP+ (SPCP+).
b. At 14 dpi, 0.5 g of symptomatic systemic leaf tissue was homogenized in a mortar with a pestle and then rub-inoculated onto the 4th, 5th, and 6th leaf of two-week old *N. benthamiana* plants. See Fig. 6 for strategy.
c. The total number of plants exhibiting necrotic lesions in upper non-inoculated leaves out of 3 independent sets of 5 replicate plants (n=15).

any serial passage thereof (Fig. 8, Table 1).

To test whether the presence of SPMV CP was due to viral vector stabilization or an increase in SPMV CP translation, RT-PCR and immunoblot analyses were performed. If the vector was being stabilized, PVX-SPCP+ should be intact and present in upper non-inoculated leaf tissues. For this, primers that crossed the SPMV CP gene insertion site were used to determine if the SPMV insert was being maintained. The primers for RT-PCR were designed with the ten 5'-proximal nucleotides of the forward and reverse primers corresponding to the vector genome, and the eleven 3'-proximal nucleotides of the forward primer and twelve 3'-proximal nucleotides of the reverse primer corresponding to the insert (Fig. 9A). Linear PVX-SPCP+ DNA (template) as a positive PCR reaction control was tested along with upper non-inoculated leaf tissue from plants inoculated with inoculation buffer or PVX-SPCP- which were used as negative controls. Based on this, only samples from PVX-SPCP+ inoculated plants gave an RT-PCR product (Fig. 9B) of the expected ca. 700 nt size. Upper non-inoculated leaf tissues that were confirmed to contain the SPMV CP by western blot analysis also showed necrotic lesions and a RT-PCR product (Fig. 9C). Taken together, the data show that SPMV CP was maintained in a PVX viral vector through serial passages in *N. benthamiana*. Since this experiment shows that SPMV CP was able to stabilize its own gene within PVX-SPCP+ (*cis*-stabilization), it was of interest to see if SPMV CP could stabilize the expression of foreign genes *in trans*.
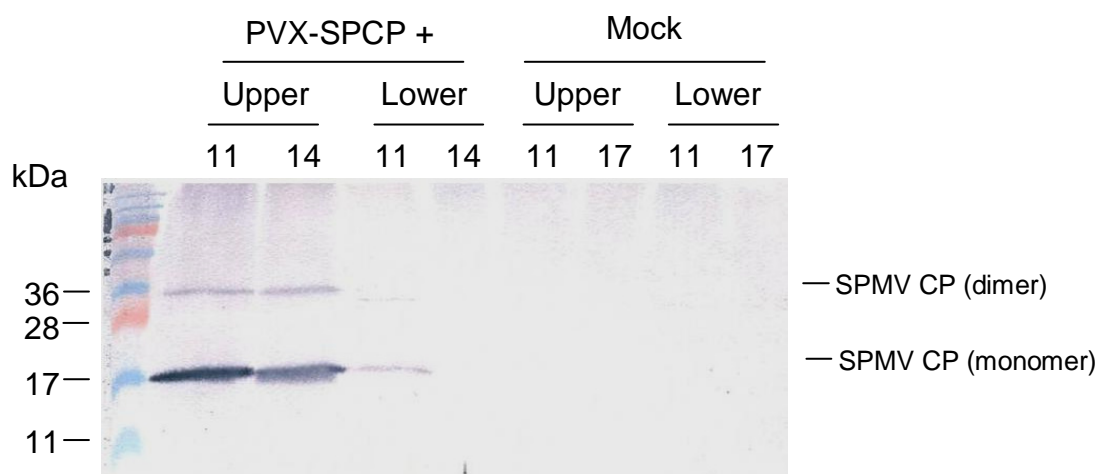
FIG. 8. Western blot analysis of upper non-inoculated leaf tissue from the third passage of plants infected with PVX-SPCP+. Two-week old *N. benthamiana* plants were inoculated with infectious transcripts of PVX-SPCP+ and monitored for the presence of indicative necrotic lesions (as shown in Fig. 6B) at 11-17 days post inoculation (dpi). Three serial passages were performed, and systemically infected leaf tissues from symptomatic plants inoculated with PVX-SPCP+ were assayed for the presence of SPMV CP using rabbit polyclonal antibodies specific for SPMV CP. Inoculation buffer (Mock) inoculated plants and PVX-SPCP- (data not shown) that had undergone three serial passages were also tested as control, and showed no SPMV CP product.
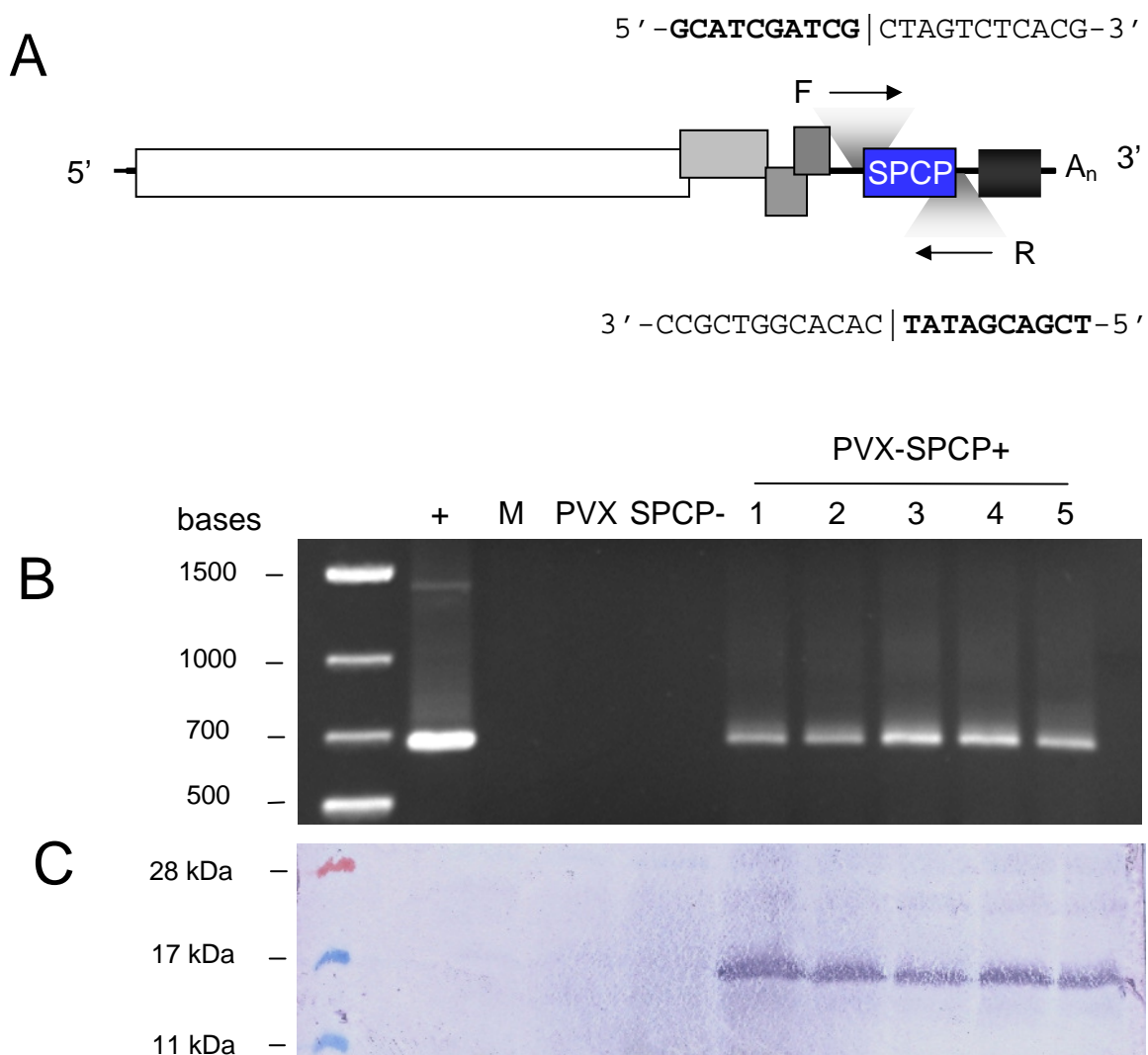
FIG. 9. RT-PCR analyses of PVX-SPCP+ infected plants. A) An RT-PCR analysis was performed using oligo-dT primers for first strand synthesis and specific primers that border the insertion site of SPMV CP. The first 10 nt of each primer corresponds to viral vector RNA (bold). B) RT-PCR products were tested for the presence of the SPMV insert on 1% agarose gels. Lanes were loaded with RT-PCR products of linear PVX-SPCP+ DNA (+), upper non-inoculated tissue from mock (M), PVX-E1S empty vector (PVX), PVX-SPCP- (SPCP-), or PVX-SPCP+ inoculated plants. The numbers represent samples collected from separate PVX-SPCP+ inoculated plants. The SPMV CP insert plus the primer ends yields a 654 nt fragment. C) Western blot analysis using rabbit polyclonal antibodies specific for the 17 kDa SPMV CP was performed using the same plant tissues as for (B).
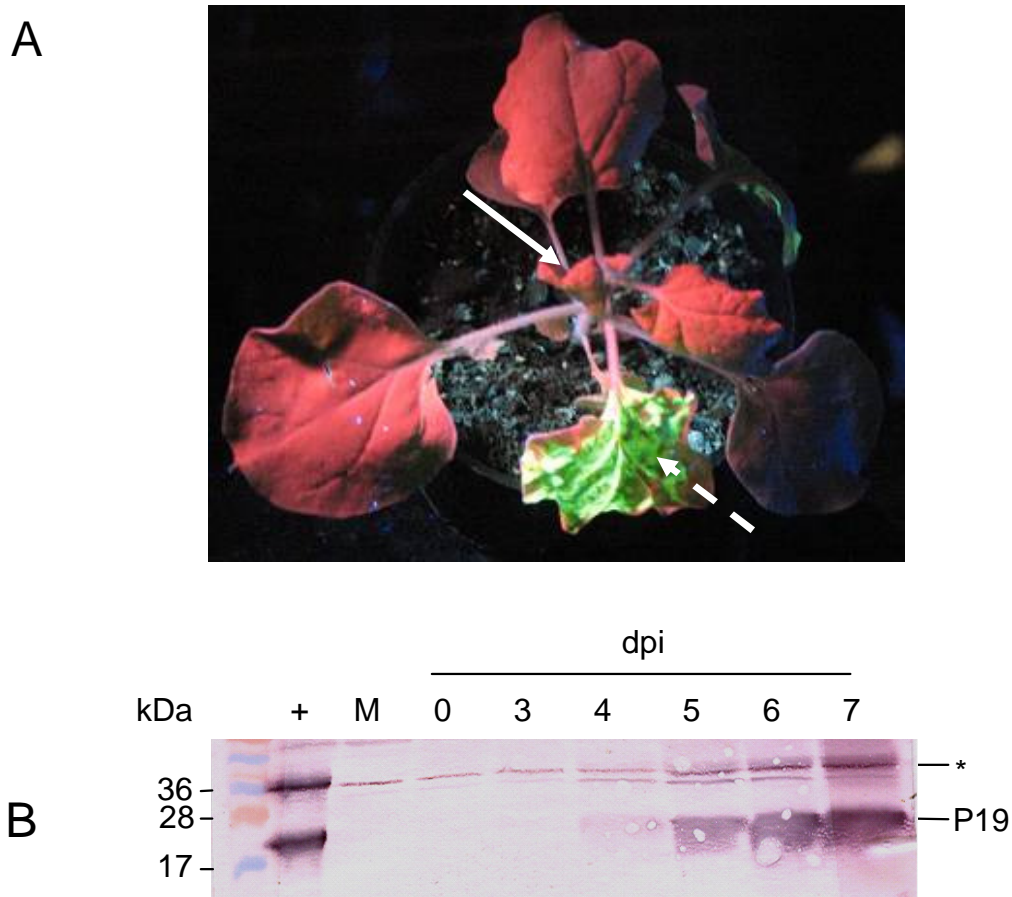
FIG. 10. TBSV-GFP infection of *N. benthamiana*. A) GFP fluoresces green under UV illumination at 488 nm in leaves of *N. benthamiana* 7 dpi with TBSV-GFP (broken arrow). Upper non-inoculated leaves (white arrow) were harvested for western blot analyses. B) TBSV-GFP infected *N. benthamiana* plants were monitored for the presence of the TBSV P19 protein, an indicator of TBSV vector presence, daily for one week using rabbit polyclonal antibodies. As control, upper leaf tissue from a plant inoculated with infectious transcripts of TBSV wild-type (+) or inoculation buffer (M) were loaded. An asterisk (*) indicates a host protein that cross-reacts with the P19 antiserum, and serves as a loading control.

***Trans*-stabilization of a GFP gene within a TBSV vector by SPMV CP expressed from PVX-SPCP+**

Based on the results from the *cis*-stabilization studies that SPCP was stabilized through serial passages in *N. benthamiana*, we developed experiments to determine if the stabilizing function of SPMV CP could be used to stabilize a GFP gene within a TBSV vector (TBSV-GFP) *in trans*. Vector construction and detailed experimental setup was described in the Materials and Methods (Chapter II). Briefly, in this experiment two-week old *N. benthamiana* plants were co-inoculated with TBSV-GFP and PVX-SPCP+ and upper non-inoculated tissues observed for the presence of GFP green fluorescence by UV illumination at 7 dpi. Control plants were inoculated with inoculation buffer, TBSV wild-type, PVX-E1S empty viral vector, PVX-SPCP+, PVX-SPCP-, TBSV-GFP plus PVX-E1S, and TBSV-GFP plus PVX-SPCP-. None of the control plants inoculated with inoculation buffer or viral vector constructs without the GFP insert showed GFP fluorescence in any tissues. In general, inoculation of *N. benthamiana* with TBSV-derived vectors results in foreign gene expression in only inoculated leaves (Scholthof, 1999a; Scholthof, Morris, and Jackson, 1993). This remains true for plants inoculated with TBSV-GFP alone or during co-infection with PVX-E1S or PVX-SPCP-, as GFP expression (fluorescence) is generally seen only in inoculated leaves of *N. benthamiana* 7 dpi (Fig. 10A). The accumulation of P19 in upper non-inoculated tissues, as determined by immunoblot analyses using P19 specific mouse-antibodies (Fig. 10B), showed that a viable TBSV viral vector is present in both inoculated and systemic tissues when plants are inoculated with TBSV-GFP. From this

A

Mock PVX PVX-SPCP- PVX-SPCP+

TBSV-GFP TBSV-GFP+PVX-SPCP- TBSV-GFP+PVX-SPCP+

B

TBSV-GFP+PVX-SPCP+

20°C 25°C
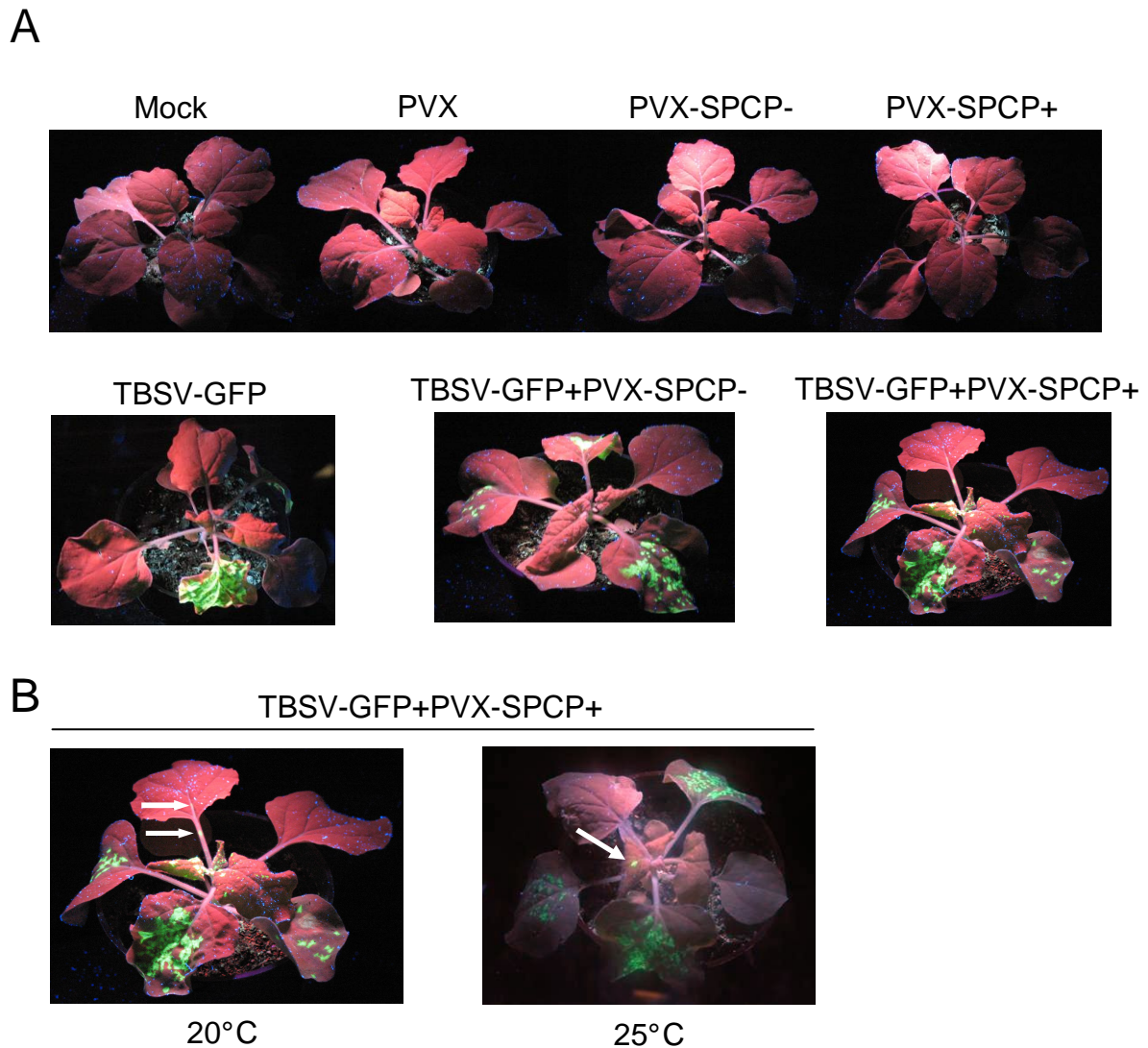
FIG. 11. *In trans* stabilization study in *N. benthamiana* using TBSV and PVX vector derivatives. A) *N. benthamiana* plants were inoculated with inoculation buffer (Mock), or PVX-E1S empty vector (PVX), PVX-SPCP-, PVX-SPCP+, TBSV-GFP, TBSV-GFP plus PVX-SPCP-, or TBSV-GFP plus PVX-SPCP+. Plants were monitored for GFP fluorescence in non-inoculated systemic tissues at 7 dpi at 20°C. B) In addition to increased GFP expression in upper non-inoculated leaves, plants inoculated with TBSV-GFP plus PVX-SPCP+ showed GFP expression in lower non-inoculated systemic tissues at 20°C (white arrows). Conducting the same experiments as in (A) at 25°C showed that green fluorescent lesions in non-inoculated upper leaf tissues were ten fold lower (see Table 2) at the higher temperature (white arrow).

and RNA analyses showing that non-essential genes, like GFP, are deleted or severely mutated during virus vector replication, it can be assumed that the resultant viral vector TBSV RNA in systemic tissues is comparable in protein expression, genome sequence, and basal functionality to that of wild-type TBSV (Scholthof, Mirkov, and Scholthof, 2002; Tzfira, Kozlovsky, and Citovsky, 2007). In addition, GFP fluorescence dampens over the course of TBSV-GFP infection. Interestingly, we observed that co-infection of TBSV-GFP with PVX-SPCP+ resulted in GFP expression in upper and lower non-inoculated leaves at 20°C, and vibrant GFP fluorescence was maintained in both inoculated and systemic leaves. This was readily observed by UV illumination at 488 nm (Fig. 11A). Expression of GFP in the upper tissues is characterized by fluorescent foci or spots that are approximately 4-8 mm in diameter and have comparable margins to the fluorescent foci found in lower inoculated tissues, though the spots in lower tissues are 8-15 mm in diameter. Of 20 co-infected plants showing GFP expression in the inoculated leaves, 19 were observed to have expression in the upper non-inoculated leaves, and a total of 225 fluorescent foci were counted in the upper tissues of 19 plants (Table 2), and fluorescence was primarily localized to secondary and tertiary veins.

Western blot analysis using a polyclonal mouse anti-P19 antibody confirmed the presence of the TBSV vector (Fig. 12) as expected based on infections with TBSV-GFP alone (Fig. 10B). To test for GFP accumulation in systemic tissues, two-week old *N. benthamiana* plants were inoculated with inoculation buffer, or transcripts of TBSV wild-type, TBSV-GFP, PVX-E1S, TBSV-GFP plus PVX-E1S, TBSV-GFP plus PVX-SPCP-, or TBSV-GFP plus PVX-SPCP+. Upper non-inoculated tissue from infected

plants was harvested 7 dpi and analyzed by immunoblot assays using mouse anti-GFP (Fig. 13A). In addition, the former tissues were also assayed for the presence of SPMV CP using rabbit anti-SPMV CP (Fig. 13B) antibodies.

In all instances where GFP fluorescence was observed in upper non-inoculated leaf tissues of plants co-infected with TBSV-GFP plus PVX-SPCP+, accumulation of both GFP and SPMV CP was observed by western blot analysis (Table 2, Fig. 13B). Interestingly, the control experiments revealed that occasionally plants inoculated with TBSV-GFP or co-infected with TBSV-GFP plus PVX-SPCP- or TBSV-GFP plus PVX-E1S (the empty vector) had a few fluorescent spots in upper tissues. However, this represents an approximately 10- to 40-fold fewer spots than those plants co-infected with TBSV-GFP and PVX-SPCP+ (Table 2). When analyzed by immunoblot assays, the same tissues from control plants were confirmed to contain the GFP protein (data not shown).

Therefore, our observation of GFP fluorescence in systemic tissues indicates that GFP gene expression or GFP gene integrity has been maintained in plants co-inoculated with TBSV-GFP plus PVX-SPCP+ (Table 2). Curiously, the GFP protein also accumulated in non-fluorescing upper leaf tissues of control plants co-infected with TBSV-GFP and PVX-E1S empty vector or PVX-SPCP- by western blot analysis (Fig. 13). This anomalous result (i.e. GFP protein, but no fluorescence) suggests that SPMV CP not only stabilizes the GFP insert within the vector, but that the gene sequence and/or functional integrity of the expressed protein is maintained. This is an important observation as it indicates that protein expression does not always result in biological

activity (Fig. 13B), but that the presence of SPMV CP in systemic non-inoculated tissues harboring TBSV-GFP allows for expression of biologically active (fluorescing) GFP.

During routine experimentation of the stabilizing effects of SPMV CP on the GFP gene insert of TBSV-GFP in *N. benthamiana*, climate control systems malfunctioned, and the temperature where plants were grown decreased from 25°C to 20°C. The drop in temperature caused a marked increase in the number of fluorescent green spots in the upper non-inoculated tissues of plants infected with TBSV-GFP plus PVX-SPCP+ (Fig. 11B). For this, a temperature study to test for environmental effects on GFP expression was undertaken. The results show that there was little difference between the number of plants showing GFP expression in upper non-inoculated tissues when the above experiments were conducted at 20°C as compared to 25°C (Fig. 11B, Table 2). Remarkably, the number of GFP fluorescent spots, as determined by UV illumination at 488 nm, was increased 10-fold in upper non-inoculated leaf tissues for plants co-inoculated with TBSV-GFP plus PVX-SPCP+ and maintained at 20°C versus plants maintained at 25°C (Table 2). These results show that stabilization of viral vectors, and thus biologically active protein expression, facilitated by SPMV CP has the potential to be optimized.

TABLE 2

Comparison of GFP expression in *N. benthamiana* at 7 days post inoculation (DPI) at 20°C and 25°C.

	Mock[b]	TBSV[b]	PVX[b]	TBSV-GFP[b]	TBSV-GFP[a] plus		
					PVX[b]	PVX-SPCP-[c]	PVX-SPCP+[c]
Plants[d] 20°C[e]	0/20 (0%)	0/20 (0%)	0/20 (0%)	3/20 (15%)	1/20 (5%)	5/20 (25%)	19/20 (95%)
25°C[e]	0/9 (0%)	0/9 (0%)	0/9 (0%)	1/9 (11%)	0/9 (0%)	3/9 (33%)	8/9 (89%)
Spots[f] 20°C[g]	0/20 (0.0)	0/20 (0.0)	0/20 (0.0)	3/20 (0.15)	1/20 (0.05)	9/20 (0.45)	225/20 (11.25)
25°C[g]	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	0/9 (0.0)	1/9 (0.11)	0/9 (0.0)	27/9 (3.00)

a. Infectious transcripts of TBSV-GFP were co-inoculated with PVX-based vectors onto the 4th, 5th, and 6th leaves of two-week old *N. benthamiana* plants (see Fig. 6).
b. Controls for this study include inoculation buffer (Mock), and infectious transcripts of TBSV, PVX-E1S empty vector (PVX), and TBSV-GFP.
c. PVX-E1S-based vectors that do not (PVX-SPCP-) or do express (PVX-SPCP+) SPMV CP.
d. 'Plants' represents *N. benthamiana* plants scored for GFP fluorescence in systemic non-inoculated tissues at 7 dpi.
e. The number of plants (numerator) observed to have green fluorescence in upper non-inoculated leaf tissues in the total number of plants observed (denominator) was used to calculate the percentage of plants with fluorescence in upper leaves, indicated in parentheses, at different temperatures.
f. 'Spots' represents the number of green fluorescent foci that were scored in systemic non-inoculated tissues of *N. benthamiana* plants at 7 dpi.
g. The number of fluorescent foci (numerator) in the total number of plants observed (denominator) was used to calculate the average number of fluorescent spots per plant, indicated in parentheses.
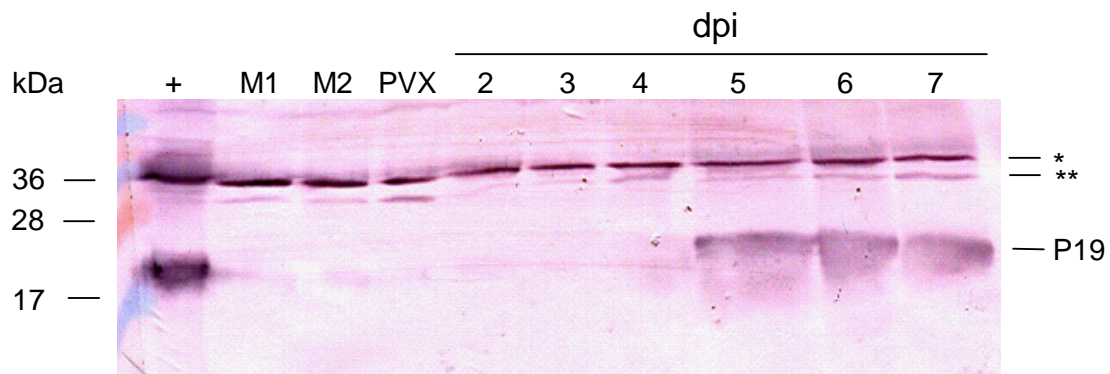
FIG. 12. Western blot analyses of P19 accumulation in upper non-inoculated leaves of *N. benthamiana* plants inoculated with TBSV-GFP plus PVX-SPCP+ at 2 to 7 days post inoculation (DPI). *N. benthamiana* plants were monitored for the presence of TBSV P19 protein from 2-7 days post inoculation. As control, upper leaf tissue from a plant inoculated with TBSV (+), mock inoculation (M1 and M2), or PVX-SPCP+ (PVX) were loaded. An asterisk (*) marks a host protein that cross-reacts with P19 serum was used as a loading control. A double asterisks (**) marks an unknown host protein.
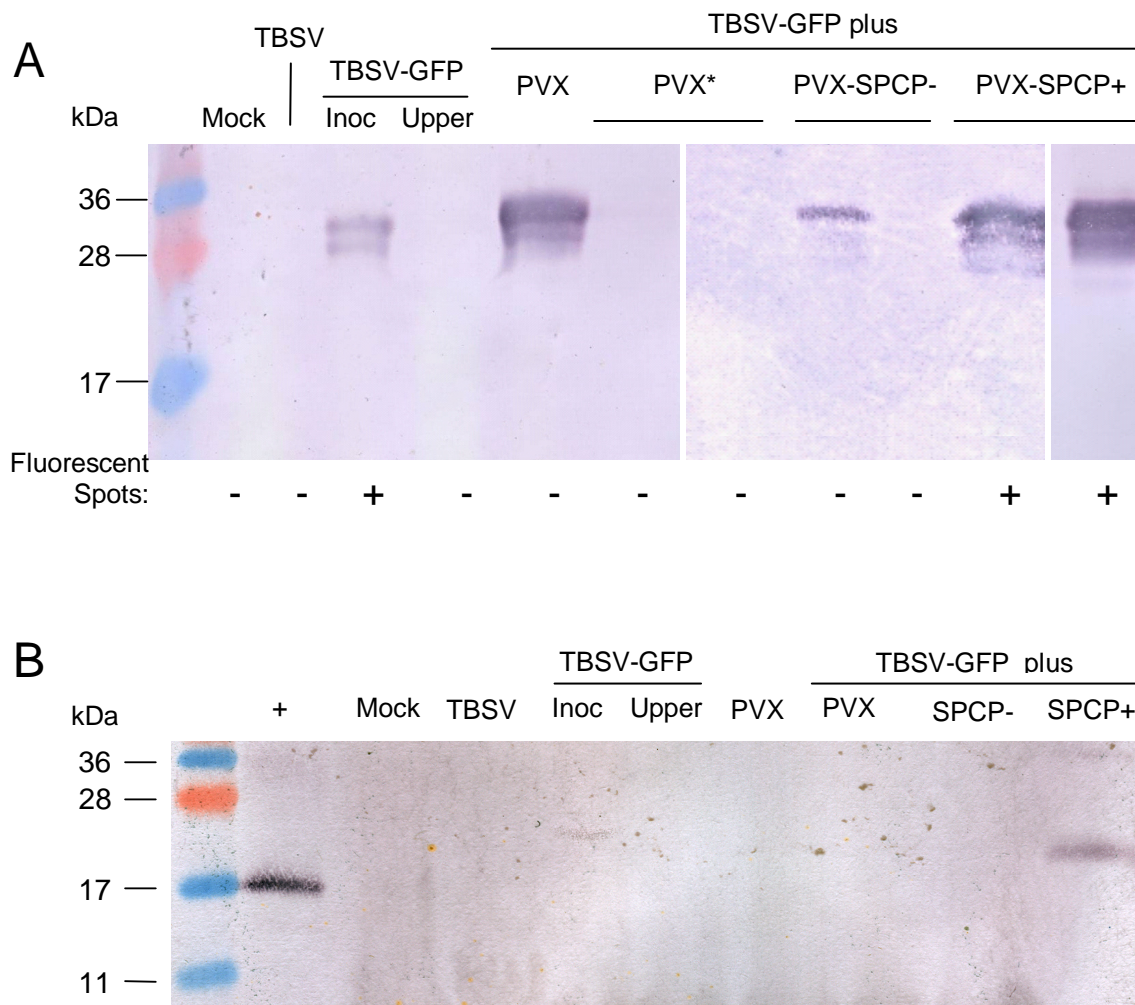
FIG. 13. GFP accumulation in upper non-inoculated leaves of *N. benthamiana* co-infected with TBSV-GFP plus PVX-SPCP derivatives. A) *N. benthamiana* plants were inoculated with inoculation buffer (Mock), TBSV, TBSV-GFP, PVX-E1S empty vector (PVX WT), TBSV-GFP plus PVX-E1S, TBSV-GFP plus PVX-SPCP-, or TBSV-GFP plus PVX-SPCP+. At 7 days post inoculation tissue was assayed for the presence of the 27 kDa GFP protein by western blot. Inoculated leaves of plants infected with TBSV-GFP were used as a positive control. Plants showing GFP fluorescence in upper non-inoculated tissues are indicated with a plus symbol (+). Western blots from two replicate experiments were combined to show that the accumulation of GFP in control plants was variable. Note that plants may accumulate GFP, but lack fluorescent spots in the absence of SPMV CP. B) Samples from (A) were used to test for the presence of the 17 kDa SPMV CP by western blot analysis. The SPMV CP band in the last lane has shifted up due to smiling. There is not equal loading of tissue samples in (A) or (B). Note that PVX-E1S (PVX*) was not co-inoculated with TBSV-GFP.

CHAPTER IV

CONCLUSIONS, FUTURE EXPERIMENTS AND FINAL REMARKS

As world populations and the cost of research increases, socially responsible biotechnology systems must be developed to address the ever growing needs of those populations at costs that are logistically feasible for researchers. These systems should include novel reagents that increase product output while being generally regarded as safe and executing sound environmental and fiscal practices (Scholthof, 2001; Scholthof, 2003). One way to address these issues is to develop biological tools that can supplement or improve existing systems. For example, pro-insulin was once purified from bovine pancreas. The process was extremely cost ineffective due to livestock maintenance and posed extreme danger to both the animals and their handlers. Additionally, feedlot waste and animal culling raised environmental and ethical concerns. One advance from the abattoir was producing proteins in *E. coli*, a bacterial system that could be easily manipulated to produce large amounts of pro-insulin in one million liter bio-reactors (Johnson, 2003; Kelley, 2001; Swartz, 2001; Zahn et al., 2008). However, this also is extremely cost ineffective as sterile environments had to be maintained to prevent contamination of the samples. Furthermore, ramp-up time takes weeks since a few milliliter samples of bacteria must be used as starting cultures and then progressively get larger to reach the one million liter limit (Johnson, 2003). Other problems with this system include energy costs related to bio-reactor usage or programmed apoptosis by the

bacteria, and/or deletions or mutations in the expressed gene, any of which can result in complete loss of a batch of cells (Kelley, 2001).

Agrobacterium tumifaciens provided an additional strategy to express a protein of interest by producing transgenic plants (An et al., 1985; Tzfira and Citovsky, 2006; Zahn et al., 2008). This system was an improvement over the fermentation process, as transgenic plants can be used for gene expression in combination with traditional methods for improving desired traits, such as breeding and nutrient supplementation. Moreover, transgenic plants produce seed that carries the gene of interest in the germ line (Lienard et al., 2007; Watrud et al., 2004). Plant systems offered an environmentally and financially sound option, as plants are relatively cheap to grow using well-established agricultural practices (Lienard et al., 2007). The caveat is that the modified genes are carried in pollen, making the genes hard to contain in the open when grown to scale (Dlugosch and Whitton; Pogue et al., 2002; Watrud et al., 2004). Additionally, large amounts of plant biomass are required due to the small amount of protein produced from individual plants (Pogue et al., 2002).

As a third alternative for large scale production of proteins, recent research has been developed that focuses on using viruses as gene expression vectors (Chung, Vaidya, and Tzfira, 2006; Zahn et al., 2008). The viruses can be used to produce foreign proteins in animals or plants without genetically modifying the host cell, as described in detail in recent reviews (Gleba, Klimyuk, and Marillonnet, 2007; Pogue et al., 2002; Scholthof, Mirkov, and Scholthof, 2002; Zahn et al., 2008). Virus replication is exponential in individual animal, insect, and plant cells and has the potential for

producing a thousand-fold more protein for the same amount of biomass compared to transgenic systems. The benefit of virus expression systems over bacterial systems is evident, as about one kilogram of desired protein can be produced in one acre of tobacco for $100, compared to $3 million for the equivalent amount of protein from a large-scale *E. coli* bio-reactor operating at capacity (Zahn et al., 2008). Using viruses as expression vectors and plants as the bio-reactors does not leave the large amount of hazardous waste that is observed with cattle feedlots or large scale fermentation reactions. Of course, viral vector systems are not flawless, as expression of the foreign gene or production of a functional protein product is lost from the vector (Gleba, Klimyuk, and Marillonnet, 2007; Pogue et al., 2002; Scholthof, Mirkov, and Scholthof, 2002). Because of this, using viruses for large scale production of proteins would greatly benefit by finding a plant host, viral vector, or molecular tool that maintains or stabilizes the gene insert within the viral gene vector.

Conclusions

The basis of my research was an earlier observation that the coat protein of satellite panicum mosaic virus might be useful as a molecular tool. This followed from previous research showing that SPMV CP has multiple biological roles in addition to cognate genome encapsidation (Desvoyes and Scholthof, 2000; Omarov, Qi, and Scholthof, 2005; Qi, 2007; Qi and Scholthof, 2008; Qiu and Scholthof, 2000; Qiu and Scholthof, 2001a; Qiu and Scholthof, 2001b; Qiu and Scholthof, 2004). For instance, SPMV CP was shown to increase PMV titers during a co-infection in *N. benthamiana*,

possibly through interactions with the PMV CP, which is known to be involved in helper virus replication (Batten, Turina, and Scholthof, 2006). In another study, green fluorescence of GFP was noted to be observed in systemic tissues of *N. benthamiana* during a co-infection of *Tomato bushy stunt virus* plus *Potato virus X* vectors expressing GFP (TBSV-GFP) or SPMV CP (PVX-SPCP+), respectively, though it was not known if SPMV CP was the causal agent of systemic GFP expression. Based on the results of the previous research, experiments were developed to test if SPMV CP could be used to stabilize PVX vectors with an SPMV CP insert *in cis*, or a TBSV vector expressing GFP *in trans*.

Cis- stabilization of a *Potato virus X* gene vector by SPMV CP

As stated in previous sections, gene inserts in PVX vectors are deleted following as few as two serial passages in *N. benthamiana* (Avesani et al., 2007). The most straightforward means to test for *cis*-stabilization by SPMV CP is to passage infected tissue through a plant host (Fig. 6) and look for evidence of the presence (or lack) of foreign protein expression. The presence of SPMV CP expressed from PVX-SPCP+ was tested for in three ways. Previous research showed that SPMV CP caused necrotic lesions in *N. benthamiana*. These small necrotic islands occurred mainly around Type I, II and III vascular tissues in non-inoculated systemic leaves (Qiu and Scholthof, 2004). The lesions only occurred when SPMV CP was present. The formation of local lesions in these tissues is likely due to a non-host response similar to that observed for other virus proteins in non-hosts (Goldberg, 1989). By direct observation, the indicative

symptoms, due to SPMV CP accumulation, allowed for quick determination of those plants retaining the SPMV CP gene. Based on these observations, SPMV CP stabilized its own 634 nt insert in a PVX vector (PVX-SPCP+) through as many as five serial passages in *N. benthamiana*. The number of plants with indicative symptoms was observed through three passages, but markedly decreased by the fourth passage. By the fifth passage, only 1 in 19 plants showed indicative symptoms (Table 1).

Multiple possibilities may exist for the formation of the necrotic lesions. For this, western blot analyses were used to confirm the presence or absence of SPMV CP in non-inoculated systemic tissues of passage plants. First, if SPMV CP was not detected where necrotic lesions were present, then SPMV CP could be responsible for a host response or signal in source tissues that induces formation of the systemic lesions (Durrant and Dong, 2004; Goldberg, 1989; Qiu and Scholthof, 2004). Second, if necrotic lesions were formed only when SPMV CP was present in the same tissues, then SPMV CP could be considered the causal agent of, as well as a viable way to monitor, PVX-SPCP+ *cis*-stabilization. Using immunoblot analyses, SPMV CP was detected in non-inoculated systemic tissues in all plants in which indicative symptoms were observed regardless of the passage, lending support to the hypothesis that SPMV CP is the stabilizing agent. However, the signal for SPMV CP in immunoblots, which was prominent through three serial passages, became increasingly difficult to detect in the fourth and fifth passages. Overexposure of western blots using upper non-inoculated tissue harvested from fourth and fifth passage plants allowed for the detection of SPMV CP (data not shown). However, the concentrations of SPMV CP that are at the boundary of the lower limit of

detection by the assay used likely reflect the eventual loss of SPMV CP expression. These results suggest that the PVX vector PVX-SPCP+ has increased stability due to the presence and activity of SPMV CP *in cis*. To investigate if SPMV CP accumulation in symptomatic systemic leaves was a result of protein mobility from source to sink tissues, RT-PCR analyses were used to test for co-localization of intact PVX-SPCP+ in these tissues(Fig. 9). Using primers that bordered the insertion site of SPMV CP, RT-PCR analyses showed the presence of the expected ca. 700 bp product. Sequencing must be performed to confirm that this product is the SPMV CP gene. Yet, direct observation and western blot analyses showed that indicative necrotic lesions and SPMV CP were present, respectively, in the same tissues that showed an SPMV-specific PCR product. RT-PCR analysis also confirmed the absence of PVX-SPCP+ in mock or PVX-SPCP-inoculated tissues, as expected. In order to get a direct comparison of stabilization, primers that border the SPCP- insert must be used on all samples to show that SPCP- is lost as passages progress.

In total, the results of the experiments with PVX-SPCP+ are significant especially in light of previous research that showed gene insert stabilization in PVX vectors decreases as insert size increases (Avesani et al., 2007; Chung, Canto, and Palukaitis, 2007; Scholthof, Mirkov, and Scholthof, 2002). In fact, prior to this study, an intact gene insert in PVX of approximately 200 nt, or one-third the size of the SPMV CP insert, was stable through only 2 serial passages and another insert approximately twice that of the SPMV CP insert was not stable in the initially inoculated *N. benthamiana* plant (Avesani et al., 2007). The experiments for stabilization by SPMV CP gave

support to the hypothesis in multiple ways. Lesions that occur only when SPCP was in the correct orientation were present through three serial passages and as many as five. Whereas plants, initially inoculated with buffer only or PVX-SPCP-, never developed symptoms or accumulated SPMV CP, as determined by immunoblot assays. However, the plants that were observed to have those lesions were positive for SPMV CP presence. Finally, passages that had necrotic lesions and were positive for SPMV CP gave an RT-PCR product that confirmed the presence of an intact PVX-SPCP+ vector. Mock or PVX-SPCP- control plants did not give an RT-PCR product, and RT-PCR analysis using primers that border the SPCP- insert still need to be performed to show the degree of stabilization that is occurring. Together, the results show that SPMV CP is able to stabilize itself within a PVX vector in *N. benthamiana*.

In trans stabilization of a *Tomato bushy stunt virus* gene vector by SPMV CP

The utility of SPMV CP as a molecular tool to stabilize plant virus gene vectors would be negligible if the only gene insert able to be stabilized was the SPMV CP gene. Therefore, and based on the results from the passage studies, it was important to determine if SPMV CP could stabilize plant virus gene vectors *in trans*. The vector to be stabilized by SPMV CP in the following experiments was TBSV-GFP. A TBSV vector was chosen because it is a well defined system and because green fluorescence in upper non-inoculated tissues of *N. benthamiana* plants allows for quick determination of TBSV-GFP presence under UV illumination (Qiu, Park, and Scholthof, 2002; Scholthof, 1999a; Scholthof, Morris, and Jackson, 1993; Yamamura and Scholthof, 2005).

To test for SPMV CP *in trans* activity, PVX-SPCP+ was co-inoculated with TBSV-GFP onto *N. benthamiana* and upper non-inoculated tissues observed for the presence of green fluorescent foci, an indicator of functional GFP accumulation. If SPMV CP is unable to stabilize TBSV-GFP, then no fluorescence would be observed in upper non-inoculated tissues. Whereas, green fluorescence should be observed in non-inoculated upper and/or systemic tissues if the GFP gene insert is stabilized by the presence of SPMV CP. Seven days post co-inoculation, green fluorescence was observed in the upper leaves of almost every plant inoculated with TBSV-GFP plus PVX-SPCP+. In addition, plants co-inoculated with TBSV-GFP plus PVX-SPCP+ showed GFP expression in lower non-inoculated systemic leaves. This is the first report of foreign gene expression in upper and lower non-inoculated leaf tissue from the TBSV-GFP vector. When plants were co-inoculated with TBSV-GFP plus PVX-E1S or PVX-SPCP- GFP fluorescence was also observed on some upper leaves, but to an equal or lesser degree than that of plants that were inoculated with TBSV-GFP alone (Table 2). Therefore, a co-inoculation with PVX-SPCP+ appeared to be able to stabilize the GFP insert when compared to control plants.

It was important to make sure that GFP fluorescence in systemic leaves was due to the *in trans* stabilization activity of SPMV CP and not the PVX vector or hyper-transportation of GFP to non-inoculated sink tissues from inoculated leaves. To test this, experiments were developed to determine if the TBSV vector, as well as SPMV CP, was in the same leaves in which GFP fluorescence was observed. For this, immunoblot

assays were performed to test for the presence of the TBSV vector, SPMV CP, and GFP in upper non-inoculated leaves.

TBSV encodes a 19 kDa protein (P19) that is responsible for pathogenicity and suppression of gene silencing (Scholthof, 2006). The presence of P19 in plant tissues of suitable hosts is an indicator of viable TBSV replication and systemic movement. Therefore, if P19 is detected in systemic tissues of TBSV-GFP inoculated *N. benthamiana* plants, then TBSV-GFP is also in those tissues. In agreement with the passage studies and the *in trans* stabilization activity hypotheses, the presence of GFP fluorescence in non-inoculated tissues, where TBSV vector and SPMV CP accumulation have been confirmed, would lend strong evidence that SPMV CP has *in trans* stabilization activity.

Immunoblot assays revealed that plants infected with TBSV-GFP, confirmed by green fluorescence in inoculated leaves, had P19 protein in upper-non-inoculated leaves (Fig. 10B). This means that the TBSV vector reached upper tissues even if no green fluorescence is observed in those tissues. From this, TBSV-GFP either lost the ability to express GFP or expresses a biologically inactive form of the protein over the course of infection. Comparable to an infection with TBSV-GFP alone, the TBSV P19 protein was detected in systemic tissues when TBSV-GFP was co-inoculated with PVX-SPCP+.

In all instances in which TBSV-GFP was co-inoculated with PVX-SPCP+ and green fluorescence was observed in inoculated leaves, green fluorescent foci also were observed in upper-non-inoculated leaves (Fig. 11). This is in contrast to control plants, in which GFP fluorescence was either not present or was present to a much lower extent

(Fig. 11, Table 2). For example, nineteen of twenty plants exhibited green fluorescent foci in systemic leaves when co-infected with TBSV-GFP plus PVX-SPCP+, whereas, only one or three plants had green fluorescent foci in systemic tissues when infected with TBSV-GFP alone or TBSV-GFP plus PVX-E1S, respectively. The results show that plants co-inoculated with TBSV-GFP plus PVX-SPCP+ more readily exhibit green fluorescence in systemic leaves, likely due to the expression and accumulation of SPMV CP. Immunoblot analyses testing for SPMV CP accumulation confirmed the presence of SPMV CP in upper tissues that were also observed to have green fluorescence (Fig. 13). SPMV CP accumulation was not observed in systemic leaves of the control plants. Taken together, these results suggest that SPMV CP presence is stabilizing the GFP gene insert within TBSV-GFP, and ultimately that SPMV CP activity works *in trans*.

As previously stated, green fluorescence was occasionally observed in systemic tissues of control plants that did not harbor SPMV CP. In instances where GFP fluorescence was observed, GFP protein was also determined to be present by western blot analyses (data not shown). Of interest is that GFP protein was also detected in upper leaf tissues of plants co-infected with TBSV-GFP plus PVX-E1S empty vector or PVX-SPCP- when no green fluorescence was observed. This indicates that the GFP protein is still being produced from TBSV-GFP in non-inoculated tissues, but that the biological activity (i.e. fluorescence) has been lost. In addition, the presence of GFP protein in these control plants was variable when GFP fluorescence was not observed in the upper leaves. The results suggest that SPMV CP presence also helps to maintain the functional integrity of the GFP gene product.

Two possibilities that explain the accumulation of GFP protein, but not fluorescence in non-inoculated tissues are viral recombination or the introduction of point mutations during replication of the TBSV-GFP genome. In viral recombination, segments of the TBSV genome or GFP gene are inserted or deleted (or both), respectively, into or from TBSV-GFP in a process known as template switching by the replicase during replication (Borja et al., 1999; Bujarski, Nagy, and Flasinski, 1994; Chung, Canto, and Palukaitis, 2007; Desvoyes and Scholthof, 2002; Rao and Hall, 1993). Fluorescence could be lost if these modifications occur in the location of the GFP gene that codes for the functional (fluorescent) part of the GFP protein. The introduction of point mutations in the GFP gene could also affect the fluorescent activity of GFP. If the mutations occur in the coding sequence of the active site of the GFP gene, this would result in loss of fluorescence. Another possibility is that point mutations in codons that lie upstream or downstream of the active site sequence could modify amino acid residues involved in protein folding. This is significant, as fluorescence of GFP is dependent on secondary structure (Crameri et al., 1996; Remington, 2006). For the purpose of developing new tools for biotechnology, maintenance of functional integrity is as important as gene insert stabilization, as expressed proteins that have lost their biological activity may be of little use, especially if that activity is the desired property of the protein product, as in GFP. For example, pro-insulin produced by a viral vector that had lost the ability to regulate blood sugar in humans would have not pharmaceutical usefulness. That SPMV CP stabilizes gene inserts within virus gene vectors and

maintains the fluorescent function of GFP *in cis* and *in trans*, it may be a viable molecular tool for utilitarian purposes.

The aforementioned *trans*-stabilization studies showing that SPMV CP contributes to GFP fluorescence in systemic tissues of *N. benthamiana* were conducted at 20°C. Earlier studies were conducted at 25°C, but during one set of experiments, the temperature dropped, resulting in the fortuitous observation that at 20°C there was a marked increase in the number of green fluorescent foci compared to plants that had been maintained at 25°C. The positive effect of lowering temperature on *in trans* stabilization exists within a narrow range. Preliminary *trans*-stabilization studies conducted at 15°C showed that number of green fluorescent foci in upper leaves was equal to or less than that of the plants grown at 25°C (data not shown). The increase in the number of green fluorescent spots at 20°C may indicate that stabilization is affected by host or viral processes that are regulated by temperature, and that those processes contribute to stabilization between 15-25°C with optimal contributions around 20°C. For example, the activity of proteins involved in host defense responses pathways (Durrant and Dong, 2004; Kaper et al., 1995; Kovalchuk et al., 2003) or the incidence of viral genome recombination (Bujarski, Nagy, and Flasinski, 1994; Simon and Bujarski, 1994) may decrease to contribute to *in trans* stabilization at lower temperatures. The increase in GFP fluorescence at 20°C suggests that stabilization of plant virus gene inserts by SPMV CP is a promising system that can be optimized, and that there may be other factors that can be manipulated to increase SPMV CP stabilization activity.

These results also suggest that activity can be altered to meet the needs of the application in which stabilization is to be used, which is a true hallmark of a utilitarian molecular tool. To compare, another useful molecular tool, *E. coli*, can be genetically altered to be temperature sensitive in order to test for effects of mutations in the areas responsible for the temperature sensitivity (Hooke, 1994). The results of stabilization studies at different temperatures in *N. benthamiana* showed that stabilization can be altered as more or less GFP was observed to fluoresce at 20°C or 25°C, respectively. Therefore, SPMV CP stabilization activity can be environmentally modified to meet the needs of *in planta* research. Future endeavors may concentrate on genetically modifying SPMV CP to alter stabilization. For example, SPMV CP could be mutated to have activity at temperatures that were previously non-permissive to stabilization, similar to the *E. coli* system. Additional genetic modifications could be used to increase the cost-benefit to biotechnology as well as research by altering the regions of SPMV CP responsible for increasing viral vector titers to further enhance viral vector replication. For this, less starting inocula or fewer applications of the viral vector would be needed to achieve the same amount of protein output. Additionally, molecular biology and plant pathology would benefit from a modifiable SPMV CP, as the effects on hosts or host processes due to attenuated or prolonged exposure of the foreign protein could be monitored and tested.

The use of PVX-SPCP+ is not without pitfalls. As shown in Table 1, stabilization of viral vectors by SPMV CP is not indefinite, though this could be argued to be beneficial, as the issue of containment is solved by loss of the gene insert (Pogue et al.,

2002). The addition of RT-PCR and protein sequence analysis still must be conducted to test for maintenance of the integrity of both SPMV CP during passage studies, as well as, GFP *in trans* stabilization studies.

It is also of great interest to find other methods to test, manipulate, or optimize viral vector stabilization. The experiments detailed above show how modified SPMV CP can be tested for changes in stability by comparing results to information gathered here on the activity of wild-type SPMV CP. Manipulations and mutations of SPMV CP have already been shown to have major effects on the biological activity of the protein (Qi and Scholthof, 2008; Qiu and Scholthof, 2001b), but at this time nothing is known of the effects of those mutations on viral vector stabilization. The goals of optimization include increasing the number of viral vectors still carrying a foreign gene insert or the level of foreign gene expression. There are multiple strategies to accomplish this goal.

Stabilization may be improved by having SPCP present in plant cells before the addition of TBSV-GFP, as plant processes needed for virus vector translation would be limited to the viral vector of interest instead of that vector *and* PVX-SPCP+. To test this, multiple *N. benthamiana* plants will be inoculated with PVX-SPCP+ and then co-inoculated on different days with TBSV-GFP to test if the level of GFP expression can be increased in systemic tissues, characterized by an increase in the number of fluorescent spots in upper and lower non-inoculated leaf tissues. If successful, these studies could be followed-up with experiments in which transgenic plants expressing SPCP are infected with TBSV-GFP. TBSV-GFP produces a functional P19 protein responsible for interception of siRNA to circumvent the RNAi gene silencing pathway

(Omarov et al., 2006; Scholthof, 2006; Scholthof et al., 1995). For this, PVX-SPCP+ is not an optimal choice for a viral vector in transgenic studies, as the SPCP gene interferes with the gene silencing suppression activity of P25, expressed by PVX, in the host (Qiu and Scholthof, 2004). To test if P25 interference is a liability to viral vector stabilization, passage studies need to be repeated using a P25 deficient PVX mutant or an alternate vector in which SPMV CP is unable to interfere with silencing suppression, such as TMV.

Also, testing viral vector stabilization by SPMV CP in different hosts could prove to be beneficial in optimizing stabilization activity. Studies have shown that different hosts have variable levels of viral vector gene insert stabilization (Seaberg, 2008). Many of the hosts tested have been solenaceous plants, and the vector used was RMJ1, a derivative of TBSV-GFP (Seaberg, 2008). RMJ1 has the portion of the CP gene that lies between the *Not*I and *Bgl*I restriction enzyme sites removed before the addition (ligation) of the GFP gene between those sites. In these studies it was shown that host factors may be playing a role in vector stabilization (Seaberg, 2008). Additionally, establishing an infection library comparing the stabilization of different viral vectors carrying SPMV CP through serial passages in several host plants should be undertaken. As an alternative, different hosts could be co-infected with TBSV-GFP plus viral vectors carrying SPMV CP. Formation of this library would provide a useful reference. For this, researchers could determine the best plant/vector combinations to use in order to meet the needs of different applications.

Future experiments

Possible mechanisms of SPMV CP stabilization of plant virus gene vectors

The major question of stabilization is the mechanism by which it occurs. I have four possible models that can be tested (Figs. 15-16), some of which have been noted earlier. In the first model, SPMV CP could be interacting with viral vector RNA to prevent template switching that results in homologous and non-homologous recombination or to prevent the introduction of point mutations in the gene insert during replication (Figs. 14-16). The fidelity of the replicase complex could be increased by interactions with SPCP, or SPCP could be interacting with host plasmodesmata to decrease modified virus filtration. This effect has been observed for geminiviruses (Gilbertson et al., 2003).

It is known that SPCP interacts with cognate RNA because SPMV exists as virions in wild-type infections of switchgrass and St. Augustinegrass, and several previous studies have shown an SPMV CP: RNA interaction *in vitro* (Desvoyes and Scholthof, 2000; Omarov, Qi, and Scholthof, 2005; Qiu and Scholthof, 2001b). What is not known is if SPMV CP interacts with non-cognate RNA. If the interaction does exist, this may show that SPMV is acting to protect non-cognate RNA. The most straightforward assay is a northwestern blot using SPMV CP and non-cognate RNA. For this, radioactively labeled transcripts of PVX, TBSV-GFP, and SPMV (as control) can be individually washed over SPCP bound to a nitrocellulose membrane followed by exposure to autoradiography film, as described previously (Desvoyes and Scholthof, 2000). If a radioactive signal is detected, then SPMV CP may be acting to shield viral
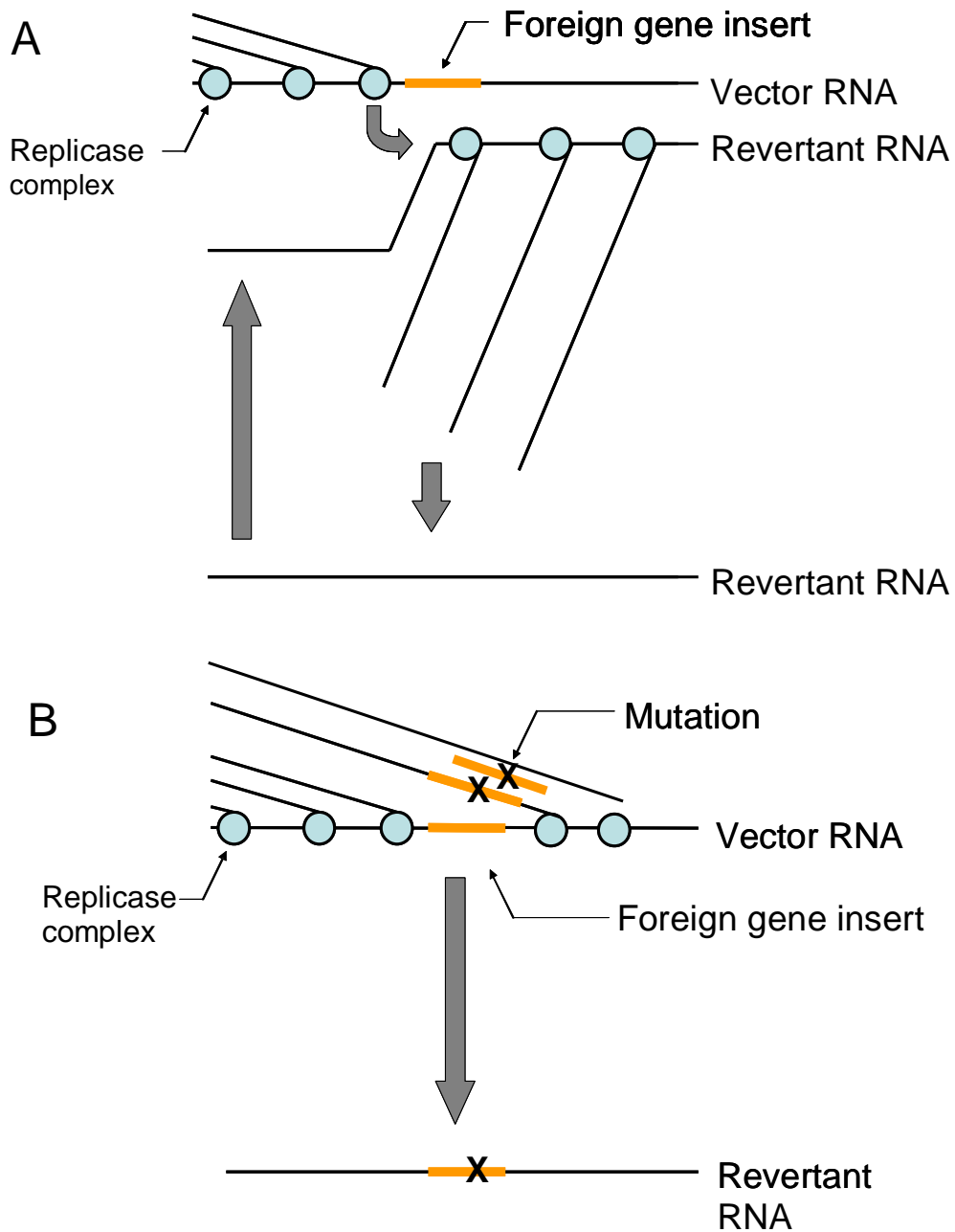
FIG. 14. Template switching and genome slippage results in the loss of gene insert expression. A) As the viral replicase (light blue circle) synthesizes a complementary strand of viral RNA (black line), it switches to another viral RNA strand (bent grey arrow) to produce a revertant viral genome (small grey arrow) that is unable to express a functional gene insert (orange bar). The revertant RNA is then available to the replicase to undergo further recombination (large grey arrow). B) Due to slippage of the viral replicase during complementary strand synthesis, mutations are introduced that abolish gene insert expression to produce revertant RNA (large grey arrow).
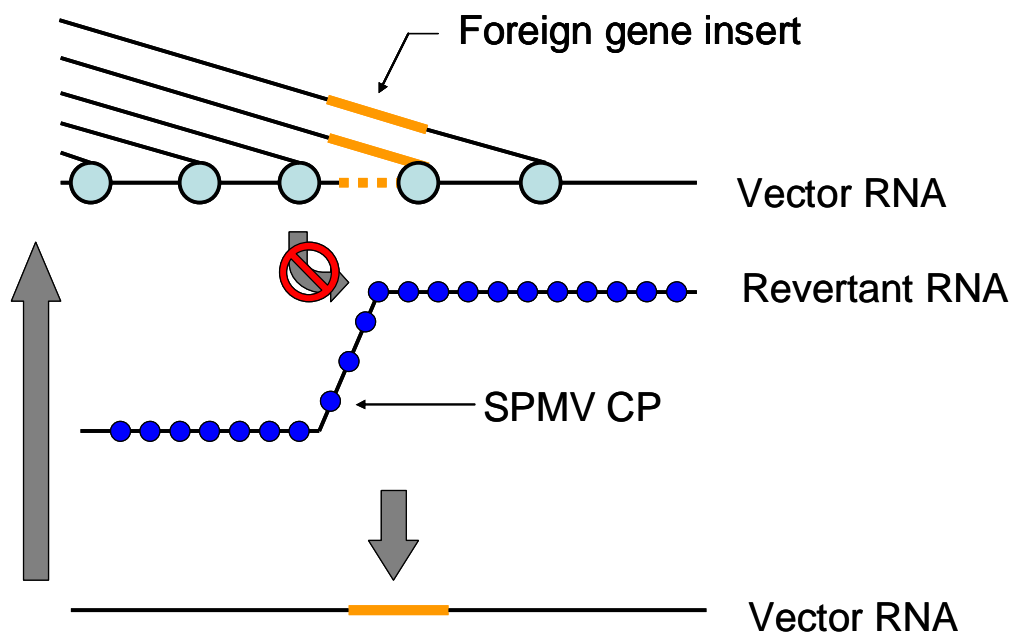
FIG. 15. Possible mechanisms of stabilization due to an SPMV CP interaction with viral vector RNA. SPMV CP (dark blue circle) interacts with viral vector RNA (black line) to prevent template switching by the viral replicase (light blue circle) to maintain an intact viral vector (small grey arrow). This occurs by preventing access to revertant viral RNA (solid black line) that has lost the ability to express a functional gene insert (orange bar) or by inducing secondary structure that is non-conducive to homologous and non-homologous recombination.
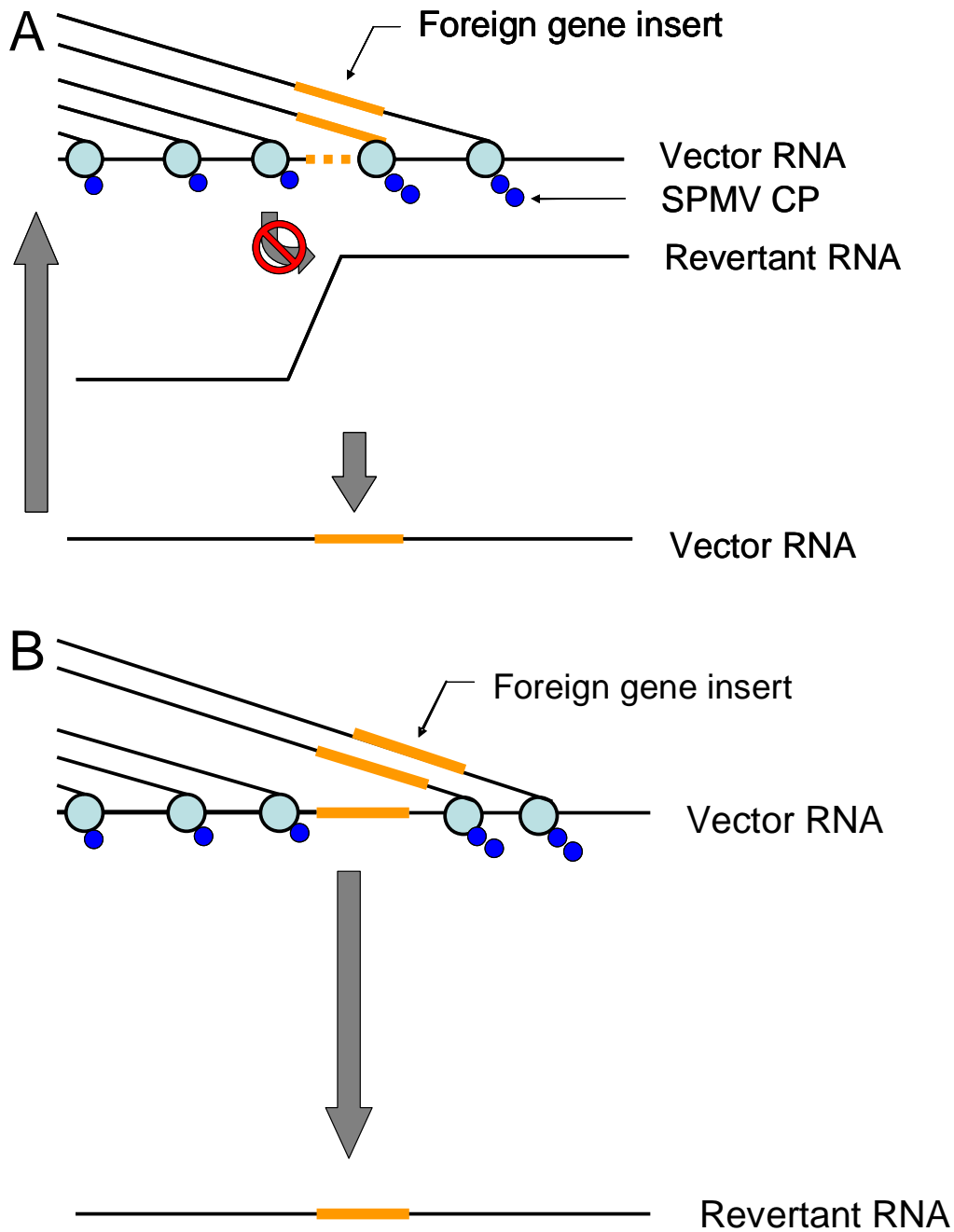
FIG. 16. Possible mechanisms of stabilization due to an SPMV CP interaction with the viral vector replicase. A) An interaction with SPMV CP monomers or dimers (dark blue circle) prevents access to revertant RNA (solid black line) unable to express a functional gene insert (orange bar) by the viral replicase (light blue circle) to stabilize the viral vector (small grey arrow). B) SPMV CP prevents the introduction of mutations that abolish expression of the gene insert by interaction with the viral replicase (large grey arrow)(see Fig. 4).

vector RNA from homologous and non-homologous recombination or creating a more stable secondary structure in the insert or viral vector RNA (Braun and Hemenway, 1992; Carpenter et al., 1995). An alternate approach would be a gel mobility shift assay. Briefly, SPMV CP would be exposed in increasing amounts to radioactively labeled viral vector RNA and then analyzed by electrophoresing the sample in a polyacrylamide gel followed by northern blot as previously described ((Desvoyes and Scholthof, 2000). If the band corresponding to the viral vector RNA in the western blot shifts up, showing an increase in molecular weight, then evidence would exist that SPMV CP interacts with viral vector RNA.

Multiple observations suggest that SPMV CP may affect an interaction of viral vector RNA with the viral vector RNA dependent RNA polymerase (RdRp). From these, a second and third model of viral vector stabilization can be formed (Figs. 15-16). First, SPMV interacts with the replicase complex of its helper virus, PMV (Batten, Turina, and Scholthof, 2006). Second, PMV titers are increased in the presence of SPMV (Scholthof, 1999b). Taken together, these data suggest that the fidelity or processivity of the replicase complex of viral vectors may also be increased by SPCP.

For the second model (Fig. 16A), two experiments can be performed to test for an SPCP-associated increase in viral vector replicase fidelity. By performing a real-time RT-PCR time-course assay using protoplasts and infected plant tissue can be used to determine the concentration of both the empty vector and the stabilized vector during the progression of an infection. Here, specific primers for sequences corresponding to the replicase complex genes, which are highly conserved, could be used to monitor the

concentration of both empty and transgenic viral vector RNA. Using primers specific for different parts of the interior of the SPCP gene would tell if the integrity of the gene is being maintained as well as give clues to any mutational "hot spots" that may exist in the CP gene, these results could be easily supported by sequencing the PCR products. The data from the vector and SPCP gene studies could then be compared. Based on the results in this thesis, my prediction is that the titer of viral vectors will be increased in the presence of SPCP as compared to the empty vector in inoculated host plants. From this, stabilization may be occurring by increasing the fidelity of the replicase complex of viral vectors.

For the third model (Fig. 16B), a test for a direct interaction between SPCP and viral vector replicases needs to be performed. The replicase proteins could be produced by *in vitro* translation and used as "bait" in a co-immunoprecipitation pull-down assay, where SPMV CP is the "prey". Here, the approach is to bind SPCP or the replicase proteins to a resin bead by use of antibodies specific for SPMV CP or the replicases. Then, exposure of the "bait-bound" bead to either the replicase proteins or SPCP would capture the prey proteins, respectively. Following the pull-down assay with western blot analysis using antibodies specific for either the replicase proteins or SPCP should show if there was an interaction. Earlier results have shown that an SPMV CP mutant R7/8 with tyrosine and leucine residues substituted for arginine residues at positions 7 and 8, respectively, of the CP is unable to bind RNA and therefore unable to form virions (Qi and Scholthof, 2008). From this, it may be possible to increase the stability of the PVX-SPCP+ construct by substitution the SPMV CP with R7/8, thereby preferentially

facilitating movement or replication efficiency. Another mutant, S130D, has an aspartate residue substituted for a serine residue at position 130 of the CP and is unable to form virions or CP dimers (Qi and Scholthof, 2008), but is movement competent in millet. Using a PVX-SPCP+ construct with an S130D mutation will provide a means to test if SPMV CP dimerization is essential for gene insert stabilization. This event, or abrogation of the effect, would limit SPCP to stabilization and away from dimerization. Both PVX-R7/8 and PVX-S130D may be a limiting factor for stabilization if the role of SPCP is primarily movement. Alternately, dimers may be critical for supporting the fidelity of viral vector replicases *in cis* or *in trans.* Based on SPCP-viral replicase interaction model (Fig. 16), the CP dimer would interact with RdRp or host encoded factors to positively affect full-length RNA accumulation. If the mechanism of stabilization involves interactions with viral vector RNA, as in the SPCP-viral RNA interaction model (Fig. 15), then R7/8 would show decreased levels of stabilization, as it cannot bind RNA (Qi, 2007). Whereas the S130D mutant may show increased levels of stabilization as it does not form virions or dimers, therefore limiting its function to RNA binding. If the mechanism of stabilization involves an interaction with viral vector replicases, as in the SPCP-viral replicase interaction model (Fig. 16), then both R7/8 and S130D mutants may show increased levels of stabilization, as the loss of RNA binding or dimerization functions may limit them to replicase interaction. If SPMV CP dimers are needed for stabilization to occur, then R7/8 would show increased levels of stabilization as its function has been limited towards replicase interaction, but S130D would show less stabilization.

Stabilization of GFP by SPMV CP *in trans* may also be due to a decrease in viral vector recombination and subsequently the generation of DI RNAs (Figs. 14A, 15, 16A). Previous work has shown that SPMV constructs unable to express the CP have increased amounts of DI formation *in planta* (Qiu and Scholthof, 2001b). Stabilization may also be occurring by increasing the fidelity or processivity of the replicase to either prevent template switching or to decrease the introduction of point mutations in the foreign gene insert during replication (Rao and Hall, 1993; Reade, Wu, and Rochon, 1999; Wang et al., 2004). RT-PCR analysis of SPCP *cis*-stabilization through serial passages indicates that SPMV is active at the level of recombination, since the gene products observed are of the expected size (ca. 650 nt) (Fig. 9B). The appearance of fluorescent and non-fluorescent GFP in non-inoculated systemic tissues suggests that the accumulation of SPMV CP prevents or abrogates the loss of fluorescence of the GFP gene product. Therefore, stabilization may be occurring by preventing the loss of the non-essential GFP gene or of point mutations resulting in loss of the fluorescence function of the GFP protein (Fig. 14). This may occur by preventing template switching or genome slippage by the viral encoded replicase of the plant virus vectors (Figs. 15-16).

In one mechanism, SPMV CP is preventing the introduction of mutations in the GFP gene at key sites responsible for fluorescence (Figs. 14B, 16B). GFP is highly dependent on a serine-tyrosine-glycine residue triad at amino acid positions 65-67 for green fluorescence, and mutations within this triad can greatly diminish GFP fluorescence as well as disrupt chromophore-dependent secondary structure (Remington, 2006). Sequencing the TBSV-expressed GFP (Fig. 5) is planned to determine if there has

been mutations in the GFP gene that results in the loss of fluorescence of the protein. If no mutations are observed, this may mean that SPMV CP interacts with a host protein(s) that interferes with GFP functionality. If mutations are detected, this would lend strong evidence for SPMV stabilization. It is also possible that both mechanisms, or modes of action, occur and that SPMV CP stabilization both decreases recombination and increases replicase fidelity (Figs. 15-16).

In addition, viral vector stabilization may include the facilitation of systemic movement of viral vectors by SPCP. Research has shown that plasmodesmata play a role in filtering out undesirable or non-cognate sequences from a plant virus (Gilbertson et al., 2003). RNA viruses, including PVX and TBSV, have proteins that interact with plasmodesmata for the purposes of transporting their genomes from cell-to-cell and/or increasing the size exclusion limit of the plasmodesmata (Ju et al., 2007; Krishnamurthy et al., 2003; Krishnamurthy et al., 2002; Mitra et al., 2003; Scholthof, 2005; Scholthof et al., 1995; Verchot-Lubicz, 2005; Verchot-Lubicz, Ye, and Bamunusinghe, 2007). Studies have shown that SPMV CP associates with the cell-wall, cytosolic, nuclear, and nucleolar fractions and may interact with Cajal bodies (Qi and Scholthof, 2008). SPMV CP also has a high affinity for cognate RNA and greatly contributes to the accumulation of SPMV RNA in systemic tissues of proso millet (Desvoyes and Scholthof, 2000; Omarov, Qi, and Scholthof, 2005). An interaction of SPMV CP and non-cognate viral vector RNA may support this hypothesis. Preliminary experiments tested movement complementation by SPMV CP during a co-infection of proso millet with PMV that lacks the ability to express the P8 movement protein (Turina, Desvoyes, and Scholthof,

2000). The data suggest that SPMV CP does not complement the cell-to-cell movement function of P8, as PMV is not found in systemic tissues of proso millet or *Brachypodium distachyon* by immunoblot analyses testing for PMV CP accumulation at 14 or 21 days post inoculation (data not shown). Alternatively, SPMV CP may contribute to systemic movement (Turina, Desvoyes, and Scholthof, 2000), so a similar approach will be undertaken using a PMV construct unable to express the P15 protein believed to be involved in systemic spread of the helper virus (Turina, Desvoyes, and Scholthof, 2000). If successful, SPMV facilitated systemic movement of its helper virus, PMV, should result in symptoms comparable to a co-infection with wild-type PMV. If movement can be restored by SPMV CP, then evidence would exist to support stabilization by an SPMV CP interaction with viral vector RNA. Furthermore, it may show that movement within a host is one of the determining factors of viral vector stability.

Final remarks

The use of viral vectors to express foreign proteins are attractive because of the rapid and high yield of gene product that can be achieved compared to other systems currently in use, especially transgenic expression (Chapman, Kavanagh, and Baulcombe, 1992; Gleba, Klimyuk, and Marillonnet, 2007; Gleba, Marillonnet, and Klimyuk, 2004; Nagyova and Subr, 2007; Scholthof, 1999a; Scholthof, Scholthof, and Jackson, 1996; Scholthof, Mirkov, and Scholthof, 2002; Tzfira, Kozlovsky, and Citovsky, 2007). One difficulty in using PVX and TBSV as viral vectors, compared to transgenic expression, has been the restriction of gene expression to the inoculated leaves. In this research we

have discovered that SPMV expressed *in cis* and *in trans* from virus vectors renders the systems permissive for systemic expression.

In all, SPMV CP was shown to stabilize its own gene within a PVX vector through multiple passages in *N. benthamiana*, showing that functional viral vector expression systems could be maintained without the need for booster or re-inoculations of plants used as viral "bio-reactors". SPMV CP was also able to stabilize GFP within TBSV-GFP *in trans*. Here, the potential usefulness of SPMV CP as a molecular tool is shown. The utility of this protein was applied to TBSV-GFP to show that SPMV CP could stabilize plant virus gene vectors *in trans*, and this may hold true for other vectors. In the future, SPMV CP may prove to be a powerful reagent in plant and virus molecular biology as well as plant pathology, agriculture, and biotechnology. Using SPMV CP, information can be gained that was previously unavailable due to the loss of the foreign gene or inaccessibility of plant tissues to that foreign gene after the onset of virus infection. Additionally, functional GFP was found in all parts of *N. benthamiana* that was co-inoculated with TBSV-GFP plus PVX-SPCP+, showing that in addition to gene insert stabilization and maintained expression, that functionality of the protein was also maintained. This is of particular importance if the activity of the gene product is the desired trait of the inserted gene. Overall, this system has the potential to result in exciting new applications for high throughput analysis for genomics, overexpression of valuable proteins for biotechnology, including bio-fuel, pharmaceutical, and crop improvement applications.

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Abstracts:

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