CHARACTERIZATION OF *POTATO VIRUS X* (PVX)

TGBp1: ROLE IN VIRUS CELL-TO-CELL

MOVEMENT AND SUPPRESSION

OF VIRUS INDUCED GENE

SILENCING

By

AMANDA R. HOWARD

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

2000

Submitted to the Faculty of the
Graduate college of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
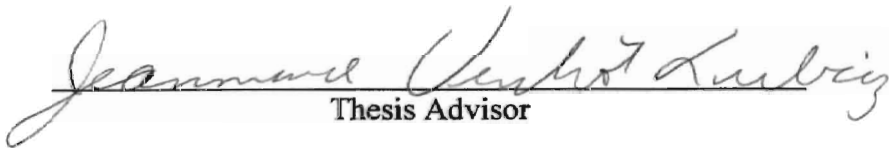December, 2003

CHARACTERIZATION OF POTATO VIRUS X (PVX)

TGBp1: ROLE IN VIRUS CELL-TO-CELL

MOVEMENT AND SUPPRESSION

OF VIRUS INDUCED GENE

SILENCING

Thesis approved:

Jeanmarie Verchot Lubicz
Thesis Advisor

Jacqueline Fletcher

Robert L Matts

Alfred Carlozzi
Dean of Graduate College

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure   Page

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Plant virus cell-to-cell movement

The ability of a virus to systemically infect a host is dependent upon its ability to move between adjacent cells and through the vasculature (Deom et al., 1992). Plant viruses move between cells via connections known as plasmodesmata (PD). PDs are plasma membrane-lined channels that are composed of a cytoplasmic annulus and a thin central core of appressed endoplasmic reticulum, known as the desmotubule (Fig. 1) (Ding et al., 1992; Lucas et al., 1993). The desmotubule is studded with proteins from which actin projections radiate and in cross section resemble the spokes of a wagon wheel. Between these proteins are microchannels through which the virus is able to traffic. PDs have a size exclusion limit (SEL; the maximum size of a molecule able to travel passively through PD) that is typically below 1 kDa (Ding et al., 1992). Many viruses can increase PD SEL to facilitate intercellular viral trafficking. The process of increasing the permeability of PD is referred to as "gating".

Plant viruses encode movement proteins (MPs), which gate PD to facilitate the trafficking of viruses between cells (Deom et al., 1990; Ding et al., 1992). The amino acid sequences of viral MPs from different virus genera seem to be highly divergent and have relatively few conserved motifs (Melcher, 1990). In spite of the poor amino acid sequence conservation, several activities have been associated with many viral MPs including the ability to bind viral nucleic acids, increase the SEL of PD, accumulate inside PD, and move from cell to cell (Angell et al., 1996; Citovsky et al., 1992; Ding et al., 1992; Fujiwara et al., 1993; Osman et al., 1992; Wolf et al., 1989).

1
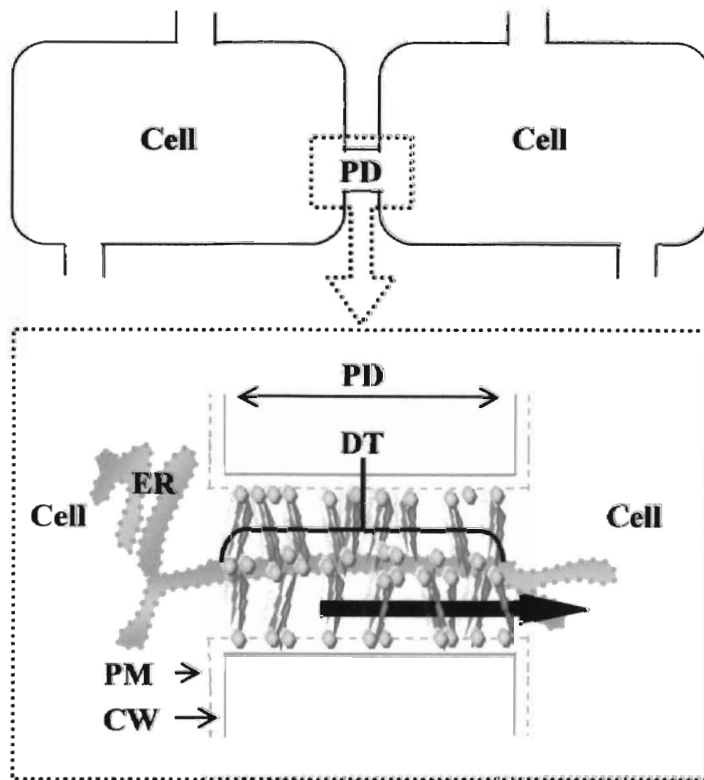
Fig. 1. Longitudinal schematic showing the structure of plasmodesmata. Top of figure shows two cells connected by plasmodesmata. Bottom of diagram shows magnified view of the plasmodesmata. The plasmodesmata (PD), endoplasmic reticulum (ER), plasma membrane (PM), cell wall (CW), and desmotubule (DT) are labeled. The ER is continuous through the PD forming the DT; the appressed ER is the core of the PD. The DT is studded with proteins from which actin filaments extend to proteins along the PM forming channels. Molecules move through these channels in the PD into the adjacent cell as indicated by the black arrow.

Three mechanisms for virus PD transport

Three mechanisms for viral cell-to-cell trafficking have been well characterized. The lack of sequence conservation among viral MPs suggests that plant viruses use a variety of mechanisms for PD transport. Thus, there are likely to be additional mechanisms that have not yet been described.

The first mechanism is typified by viruses whose MPs are related to the TMV MP. These are members of at least 12 virus genera, including *Tobra-*, *Cucumo-*, *Diantho-*, *Bromo-*, *Alfamo-*, *Furo-*, *Tenui-*, *Tobamo-*, *Begomo-*, *Umbra-*, *Tombus-*, and *Rhabdovirus* (Melcher, 2000). These viruses do not require the viral coat protein (CP) or encapsidation for PD transport, but move as ribonucleoprotein complexes consisting of viral single-stranded RNA and MP (Ding et al., 1995; Melcher, 2000). These MPs form multimers that complex with viral RNAs and shuttle them through PD. The most recent model (Fig. 2A) indicates that the TMV MP associates with viral RNA following its replication (McLean et al., 1995). Since viral replication and translation occur along the ER, the TMV MP is associated with the ER early in infection (Heinlein et al., 1998; Reichel and Beachy, 1998). The TMV MP ribonucleoprotein complex associates with the cytoskeleton and uses the microtubule and actin networks as a track to move toward and through PD (Citovsky et al., 1992; Heinlein et al., 1998; McLean et al., 1995). The viral MP increases the PD SEL to allow movement of the ribonucleoprotein complex into the adjacent cell (Fujiwara et al., 1993; Waigmann et al., 1994).

The second category includes viruses whose MPs form tubules extending between cells (Melcher, 2000; van Lent et al., 1991). Tubule-forming viruses include members of the genera *Nepovirus*, *Comovirus*, *Caulimovirus*, *Badnavirus*, and *Tospovirus* (Melcher, 2000). Virion particles, which are viral nucleic acid encapsidated by CP, move through

3

these tubules between adjacent cells (Fig. 2B) (Kasteel et al., 1997; van Lent et al., 1991; Wellink and van Kammen, 1989).

Third are the potyviruses, which do not depend on a single MP, but utilize several viral proteins that are crucial to other aspects of the virus life cycle, such as CI, VPg, and CP (Carrington et al., 1998; Carrington et al., 1996). CI is a RNA helicase required for viral replication (Eagles et al., 1994; Klein et al., 1994), and in cytological studies is associated with viral RNA and CP in cone-shaped projections that are proximal to PD (Rodriguez-Cerezo et al., 1997). These projections might promote movement of either viral particles or ribonucleoprotein complexes through PD. The VPg (virus protein genome-linked) is a protein linked to the 5' end of the genome that is present in encapsidated particles and is essential for virus replication. Because both CP and VPg are important for virus movement, the current model suggests that CI projections form a channel into the plasmodesmata through which virion particles are transported (Fig. 2C) (Carrington et al., 1998; Carrington et al., 1996).

Cell-to-cell movement of triple gene block-containing viruses

Hordei-, potex-, carla-, and *benyviruses* contain an evolutionarily conserved genetic module named the "triple gene block" (Memelink et al., 1990; Morozov et al., 1987; Morozov et al., 1989; Skryabin et al., 1988). This module consists of three partially overlapping reading frames (ORFs) that encode viral movement proteins termed TGBp1, TGBp2, and TGBp3, which are all required for virus cell-to-cell movement (Beck et al., 1991; Huisman et al., 1988; reviewed in Morozov and Solovyev, 2003).

The potexvirus TGBp1 can increase PD SEL and can move from cell to cell (Angell et al., 1996; Lough et al., 1998; Yang et al., 2000;). TGBp1 represents superfamily 1 NTPases/helicases and has been shown *in vitro* to bind viral nucleic acids

4

and to have GTPase/ATPase activities (Kalinina et al., 1998; Morozov et al., 1999; Rouleau et al., 1994). The superfamily 1 helicases have two spatially distinct functional domains known as subdomain 1A and subdomain 2A (Morozov et al., 1999). Subdomain 1A is comprised of four amino acid sequence motifs (I-IV) that are responsible for the NTPase activity. Subdomain 2A likely contributes to conformational changes related to coupling ATPase and RNA binding (Morozov et al., 1999). Mutations in subdomain 1A of the *Barley stripe mosaic hordeivirus*, *Beet necrotic yellow vein benyvirus*, and *White clover mosaic virus* (WClMV) *potexvirus* TGBp1 proteins disrupted ATPase activity, plasmodesmata gating, and RNA trafficking (Bleykasten et al., 1996; Donald et al., 1997; Lough et al., 1998), suggesting that ATPase activity is necessary for virus movement. Mutations in subdomain 2A might putatively disrupt viral protein-protein interactions (Morozov et al., 1999).

TGBp2 and TGBp3 are membrane-binding proteins. The potexvirus TGBp2 has two membrane-spanning domains while TGBp3 has one membrane-spanning domain. The hordeivirus and potexvirus TGBp2 and TGBp3 proteins were shown to be associated with the ER during virus infection (Solovyev et al., 2000; Krishnamurthy et al., 2003; Mitra et al., 2003). Mutational analysis also indicates that the central domain of TGBp2 interacts with TGBp1. When the transmembrane domains of TGBp2 and TGBp3 were mutated viral cell-to-cell movement was eliminated (Krishnamurthy et al. 2002; Krishnamurthy et al., 2003; Mitra et al., 2003). Thus, MP-ER association is important for virus movement.

Potexviruses, unlike other triple gene block containing viruses, require the viral CP for cell-to-cell movement (Beck et al., 1991; Chapman et al., 1992; Forster et al., 1992). The *Potato virus X* (PVX) CP accumulates inside PD but does not induce PD

5

gating (Oparka et al., 1996; Santa Cruz et al., 1998). A model for potexvirus cell-to-cell movement has been proposed based on studies of *White clover mosaic virus* (WClMV) (Lough et al., 1998) (Fig. 2D). In this model viral single-stranded (ss) RNA, TGBp1, and CP form a ribonucleoprotein complex. TGBp2 and TGBp3 serve to anchor the ribonucleoprotein complex to the ER. In this model either the ER or cytoskeleton serves as a track for the ribonucleoprotein complex to traffic toward the PD (Lough et al., 1998). TGBp1 then induces PD gating and the complex moves through into the adjacent cell.
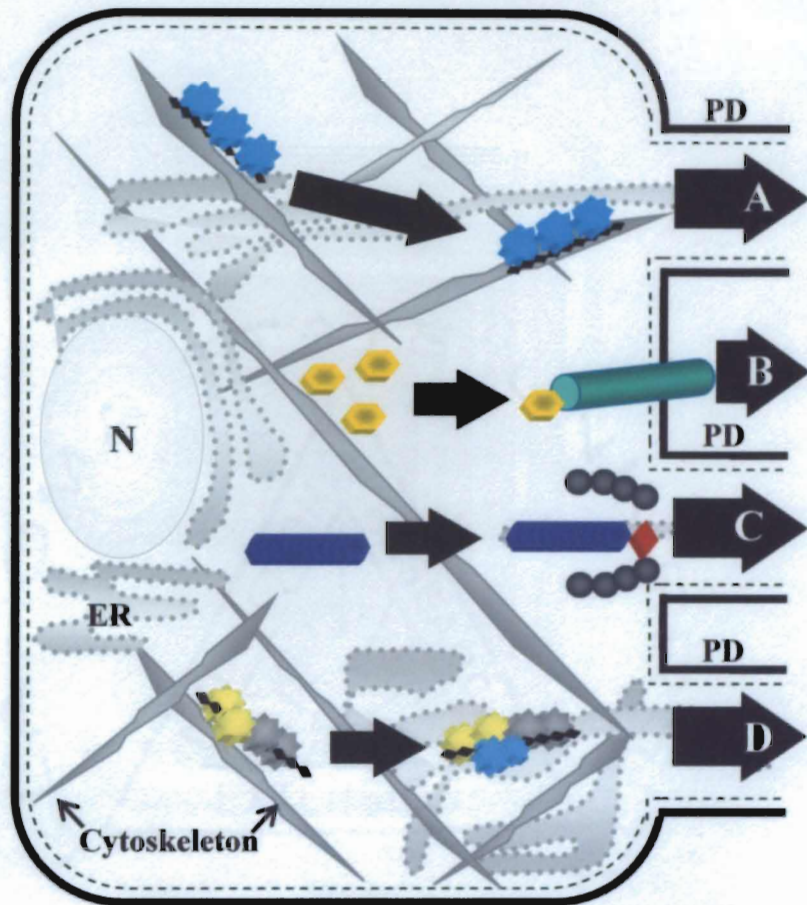
Fig. 2. Diagram of a cell depicting models of virus cell-to-cell movement. The endoplasmic reticulum (ER) (gray, dotted folds), cytoskeleton (gray, jagged structures), nucleus (N), and plasmodesmata (PD) are labeled. The large black arrows indicate virion or protein/nucleic acid complex movement into the adjacent cell. (A) TMV movement protein (blue sun-shape) binds to viral RNA (black corrugated line) and moves along the cytoskeleton to the PD; the MP/vRNA complex moves through the PD into the adjacent cell by associating with actin. (B) MPs complex to form tubules that extend through the cell wall (green cylinder) through which the virion particles (yellow hexagons) move into the adjacent cell. (C) Potyviral virions (blue) associate with the VPg (red diamond) and CI (gray spheres), which form a channel in the PD through which the virions move into the adjacent cell. (D) TGBp1 (yellow sun-shape) and CP (gray sun-shape) associate with the viral RNA. The TGBp1/CP/vRNA complex associates with TGBp2/TGBp3 at the ER and the entire complex moves into the adjacent cell.

Potato virus x (PVX)

Research in our laboratory focuses on PVX, which is the type member of the genus *Potexvirus*, and is a (+)-strand RNA virus that encodes five open reading frames (ORFs) (Huisman et al., 1988). The gene nearest the 5' end of the PVX genome encodes a 166-kDa replicase protein (Fig. 3A) (Davenport and Baulcombe, 1997). Adjacent to the viral replicase is a genetic module of three partially overlapping open reading frames, termed the "triple gene block", which is required for virus cell-to-cell and vascular transport (Beck et al., 1991; Huisman et al., 1988). The PVX triple gene block proteins (named TGBp1, TGBp2, and TGBp3) have molecular masses of 25-kDa, 12-kDa, and 8-kDa, respectively (Fig. 6A) (Beck et al., 1991; Koonin and Dolja, 1993; Verchot et al., 1998). The CP gene (ORF 5), which lies near the 3' end of the genome, is required for encapsidation and viral intercellular trafficking (Fig. 3A) (Chapman et al., 1992; Forster et al., 1992).

Three subgenomic RNAs (sgRNAs) are required to express the triple gene block and CP ORFs. TGBp1 is expressed from sgRNA1, TGBp2 and TGBp3 are overlapping ORFs expressed from sgRNA2, and CP is expressed from sgRNA3. An infectious clone of PVX (named pPVX204) that contains the green fluorescent protein (GFP) gene inserted into the viral genome adjacent to a duplicated CP sgRNA promoter (Fig. 3B) was prepared previously (Verchot al., 1995). GFP expression was used to monitor virus cell-to-cell and vascular transport (Baulcombe et al., 1995; Verchot et al., 1995; Yang et al., 2000; Krishnamurthy et al., 2003).

Plasmids were prepared previously that contain the GFP gene fused to TGBp1, TGBp2, or TGBp3 ORFs. These plasmids were bombarded to *N. benthamiana* and *N. tabacum* leaves. To study protein cell-to-cell movement, the spread of GFP fluorescence from the initially bombarded cell was monitored using epifluorescence microscopy (Yang et al. 2000; Krishnamurthy et al., 2002; Krishnamurthy et al., 2003). Research thus far suggests that the requirements for protein cell-to-cell movement are host specific (Krishnamurthy et al. 2002; Krishnamurthy et al., 2003). In *N. benthamiana* leaves, each fusion protein moved from cell to cell. In *N. tabacum* leaves GFP:TGBp1, but not GFP:TGBp2 or GFP:TGBp3, moved between adjacent cells (Krishnamurthy et al., 2002).

Transgenic *N. tabacum* expressing the PVX TGBp1, TGBp2, TGBp3, the combined TGBp2/TGBp3, or CP were also used to study protein cell-to-cell movement. Plasmids were bombarded to the transgenic *N. tabacum* leaves to determine if interactions among the triple gene block proteins can alter protein cell-to-cell movement. In TGBp1-expressing transgenic *N. tabacum*, GFP:TGBp2 or GFP:TGBp3 could move from cell to cell (Krishnamurthy et al., 2002). However, TGBp1 could not move in CP-expressing or in TGBp2/TGBp3-expressing transgenic tobacco (Krishnamurthy et al., 2002). Taken together, these data indicate that TGBp1 can interact with each of the other PVX proteins and these interactions may regulate protein cell-to-cell movement. However, we do not know specifically how these interactions relate to virus movement. These data also suggest that the model presented in Fig. 2D by Lough et al. (1998) may be incomplete.

A PVX genome

Triple gene block
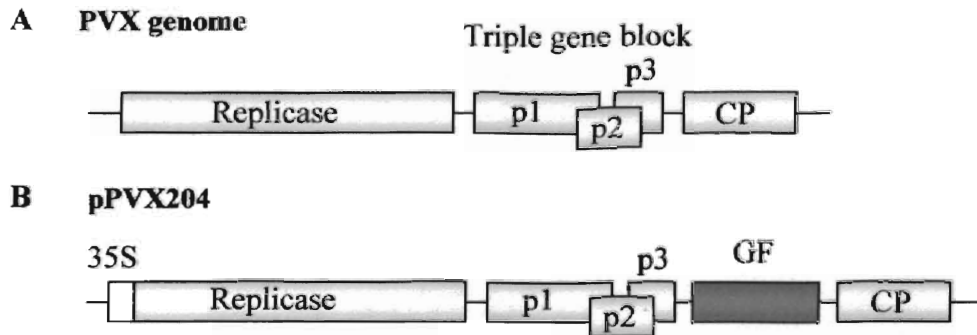
B pPVX204

Fig. 3. PVX genome and pPVX204 construct used in studies of PVX cell-to-cell and systemic movement. PVX ORFs are shown in light gray. (A) The PVX genome consists of 5 ORFs. MPs TGBp1, TGBp2, and TGBp3 comprise the highly conserved triple gene block. (B) pPVX204 is a plasmid containing a cDNA copy of the PVX genome adjacent to the *Cauliflower mosaic virus* (CaMV) 35S promoter (white box). The dark gray box is GFP. pPVX204 can be rub-inoculated to leaves or biolistically delivered to leaves to initiate virus infection.

Virus induced gene silencing and PVX TGBp1

Virus induced gene silencing (VIGS) is initiated in transgenic plants by a virus that shares nucleic acid sequence homology with the transgene (Vaucheret et al., 1998). The sequence homology between the transgene and viral RNAs induces an RNA degradation pathway causing a reduction in the steady-state levels of both transgene mRNAs and viral RNAs (Baulcombe 1996). This mechanism protects plants from systemic infection by RNA viruses and plants often recover from virus infection (Vaucheret et al., 1998; Waterhouse et al., 1998).

In recovered plants severe symptoms are often seen on inoculated and first systemic leaves, while the upper leaves are often symptom-free (Ratcliff et al., 1997). A similar "recovery" phenomenon was reported in transgenic tobacco containing the *Tobacco etch virus* (TEV) CP gene (Dougherty et al., 1994). Inoculated transgenic leaves showed symptoms caused by the *Potyvirus Tobacco etch virus* (TEV) whereas the upper leaves were symptom-free (Dougherty et al., 1994)

Moreover, in experiments using TEV CP-transgenic plants inoculated with TEV, northern analysis revealed that virus accumulation in the upper leaves was reduced (Dougherty et al., 1994; Lindbo et al., 1993). Recovered leaves were resistant to secondary inoculation with the homologous virus but were susceptible to infection by heterologous viruses (Dougherty et al., 1994; Ratcliff et al., 1997). These experiments led to the characterization of the mechanism of gene silencing in plants.

VIGS has been explored in studies using the PVX.GFP and the *Tobacco rattle tobravirus* (TRV) infectious clone. GFP was introduced into either the PVX or TRV genomes (Ratcliff et al., 2001; Ruiz et al., 1998). GFP expression is used to monitor virus accumulation in upper non-inoculated leaves. In classic experiments, PVX.GFP or

11

TRV.GFP was inoculated to GFP-expressing transgenic *N. benthamiana* (Ratcliff et al., 2001; Ruiz et al., 1998). In both cases GFP expression disappeared throughout the plant.

The TRV infectious clone has also been used to silence plant endogenous genes and may be useful in genetic studies (Ratcliff et al., 2001). Since TRV is one of a few plant viruses that can enter meristematic cells, the TRV vector may be a tool for analyses of meristematic gene expression in plants other than *Arabidopsis* or *Antirrhinum* (Ratcliff et al., 2001). To demonstrate the power of TRV in these types of studies, the meristematic gene *Nicotiana LFY*, which is expressed in flower primordia and is the tobacco homologue of *Arabidopsis LFY*, was inserted into the TRV infectious clone. Plants inoculated with this version of TRV had flower defects that resembled defects reported in *Arabidopsis lfy* mutants and did not exhibit symptoms of virus infection (Ratcliff et al., 2001).

The recovery phenomenon has also been observed in tobravirus infected plants. These plants show a natural recovery from virus infection that does not require a homologous transgene. In recovered plants a severe disease is often seen on inoculated and first systemic leaves while the upper leaves are often symptom-free (Ratcliff et al., 1997). This suggests that gene silencing is a natural part of virus infection. Plant viruses may trigger an RNA-mediated defense mechanism in plants that is manifested as a reduction in the steady-state levels of viral RNAs.

In light of what we now know about gene silencing and the recovery phenomenon, the ability of viruses to infect plants may require plant viruses to suppress VIGS. Voinnet et al. (1999) used transgenic *N. benthamiana* plants expressing GFP to determine if plant viruses suppress gene silencing. In this system, *Agrobacterium tumefaciens* carrying a binary Ti plasmid vector that contains a 35S-GFP cassette was

infiltrated into a mature leaf of a GFP transgenic *N. benthamiana* plant (Voinnet et al., 1999). Within 20 days post inoculation GFP expression was shut off throughout the plant. Then a challenge virus was used to inoculate the silenced plant. In silenced plants inoculated with the nepovirus *Tobacco black ringspot virus* (TRV), GFP expression was not restored. However in silenced plants inoculated with TRV, gene silencing was suppressed and GFP expression was restored. The TRV gene product that suppresses gene silencing has not yet been reported.

The mechanism for gene silencing has been revealed in the last few years. We now know that the mechanism is essentially a double-stranded RNA degradation mechanism that is triggered and functions systemically (Fig. 4). Often transgene or viral RNAs are reverse transcribed producing dsRNAs. An enzyme known as DICER degrades the large dsRNAs into small dsRNAs (sRNAs) that are approximately 21-25 nucleotides in length. These sRNAs are incorporated into a RNA interference specificity complex (RISC) that targets other RNA species within the cell sharing sequence similarity with the dsRNA (Fig. 4). A systemic silencing signal is produced that spreads throughout the plant initiating silencing in other tissues. The nature of this systemic signal is still unknown (Baulcombe, 2001).

dsRNA

Dicer

21-25 nt sRNAs
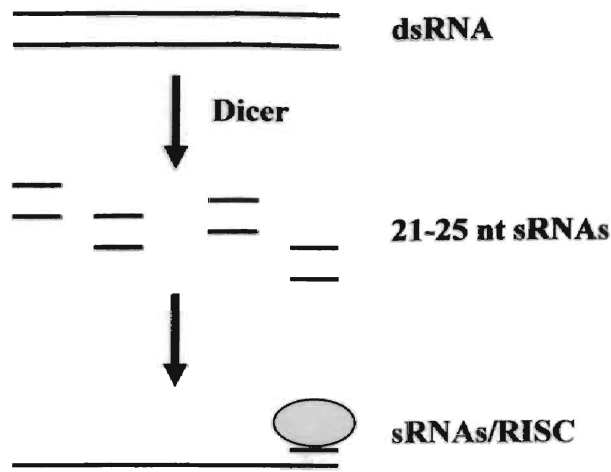
sRNAs/RISC

RISC degrades transgene/viral mRNA

Fig. 4. A basic schematic of the VIGS mechanism in plants. Gray circle is RISC. dsRNA is degraded by Dicer generating sRNAs. These sRNAs guide the RISC complex for the degradation of both transgene and viral mRNA.

A number of plant virus encoded proteins are now known to suppress gene silencing by targeting different points in the pathway. The pattern and degree of suppression is specific to the suppressor protein (Voinnet et al., 1999). For example, the *Potato virus Y* (PVY) helper-component protease (HC-Pro) can suppress gene silencing in old and new leaves, while the *Cucumber mosaic virus* (CMV) 2b protein blocks gene silencing only in newly developed leaves (Brigneti et al., 1998). Another example of suppression pattern is typified by vein centric suppression in newly emerged leaves by the p19 protein of *Tomato bushy stunt virus* (TBSV) (Voinnet et al., 1999). The PVX TGBp1 protein is a silencing suppressor that inhibits spread of the silencing signal outside of the inoculated leaf (Voinnet et al., 2000).

Research Objectives

PVX TGBp1 is essential for virus cell-to-cell movement; however, the specific activities of TGBp1 that contribute to virus movement have not been characterized. TGBp1 gates PD and is an RNA helicase and suppressor of gene silencing. It is unknown which of TGBp1 function(s) is/are most essential for virus cell-to-cell movement. It is also unknown which TGBp1 amino acid sequence domains are involved in virus and protein cell-to-cell movement, protein-protein interactions, and silencing suppression.

Experiments were conducted to further characterize the activities of TGBp1 in virus cell-to-cell movement. This study had the three following objectives:

1. Determine if TGBp1 is the primary gating factor for virus cell-to-cell movement.

2. Identify TGBp1 amino acid sequences essential for virus and protein cell-to-cell movement as well as protein-protein interactions.

3. Determine the TGBp1 amino acid sequences necessary for the suppression of gene silencing.

15

CHAPTER II

POTATO VIRUS X TGBp1 IS THE PRIMARY PLASMODESMATA GATING FACTOR

ABSTRACT

Potato virus X (PVX) TGBp1, TGBp2, TGBp3, and coat (CP) proteins are required for virus intercellular movement. Plasmids containing the green fluorescent protein (GFP) gene fused to PVX TGBp1 or CP genes were bombarded to tobacco leaves, and GFP was used to monitor protein intercellular movement. Intercellular movement of GFP:TGBp1 was observed in leaves taken from four solanaceous species, while GFP:CP moves only in *Nicotiana benthamiana* leaves. In a previous report GFP:TGBp2 and GFP:TGBp3 moved between cells in *N. benthamiana* leaves but were restricted to single epidermal cells in leaves taken from five other plant species (Krishnamurthy et al., 2003). Thus, the results of PVX experiments conducted in *N. benthamiana* often vary from results obtained in other plant species. Fluorescent dextrans (F-dextrans) were injected into mesophyll cells of non-transgenic and PVX TGBp1-transgenic *N. tabacum* leaves to determine if TGBp1 affects plasmodesmata (PD) size exclusion limit (SEL). The 9.5-kDa F-dextrans moved between cells in TGBp1-transgenic leaves but was restricted to single cells in non-transgenic leaves. These data combined with previously published reports indicate that TGBp1 is the primary PVX factor that moves between cells and induces plasmodesmata gating in more than one PVX host species.

INTRODUCTION

As mentioned previously, Potexviruses contain three partially overlapping open reading frames termed the "triple gene block"(TGB) that encode proteins called movement proteins (MP) that are required for virus cell-to-cell movement (Beck et al., 1991; Huisman et al., 1988). The potexvirus coat protein (CP) is also required for virus cell-to-cell movement (Forster et al., 1992; Santa Cruz et al., 1998). In the case of *Potato virus X* (PVX) these three proteins are named TGBp1, TGBp2, and TGBp3.

The ability of the potexvirus TGBp1, TGBp2, TGBp3, and CP to move from cell to cell and increase PD SEL has been studied using the biolistic delivery and microinjection techniques. In several reports TGBp1 was indicated as the factor responsible for PD gating (Angell et al., 1996; Lough et al., 1998; Krishnamurthy et al., 2002), and in other reports TGBp2 was identified as a factor inducing plasmodesmata gating (Fridborg et al., 2003; Tamai et al., 2001). These reports differed in their results, most likely because experiments were conducted in different *Nicotiana* species using different techniques. These conflicting results have led to some confusion as to which potexvirus proteins induce plasmodesmata gating and/or move through PD. To clarify some of the confusion, in two recent reports we have undertaken experiments using biolistic bombardment to deliver plasmids expressing GFP fused to TGBp1, TGBp2, TGBp3, or CP open reading frames to a variety of PVX host species. In one study, plasmids were biolistically delivered to *Nicotiana benthamiana* and *N. tabacum* leaf epidermal cells (Krishnamurthy, 2002). Cell-to-cell movement of GFP:TGBp1, GFP:TGBp2, GFP:TGBp3, and GFP:CP was observed in *N. benthamiana* leaves, but only GFP:TGBp1 moved between adjacent cells in *N. tabacum* leaves (Krishnamurthy et al., 2002). To expand this work, plasmids expressing GFP:TGBp2 or GFP:TGBp3 were

17

biolistically delivered to six different PVX host species as a means to compare protein activities in a broader range of PVX host plants (Krishnamurthy et al., 2003). GFP:TGBp2 and GFP:TGBp3 each moved from cell to cell in *N. benthamiana* leaves but were restricted primarily to single cells in *N. tabacum, N. clevelandii, Lycopersicon esculentum, Chenopodia quinoa,* and *Gomphrena globossa* (Krishnamurthy et al., 2003).

Microinjection studies conducted in different laboratories also used different host plants and sometimes produced conflicting data. In one study, fluorescence-labeled dextrans (F-dextrans) of various sizes were injected into non-transgenic or transgenic *N. benthamiana* expressing *White clover mosaic potexvirus* (WClMV) TGBp1, TGBp2, TGBp3, CP, or the combined TGBp2 + TGBp3 proteins (Lough et al., 1998). Large 10-kDa F-dextrans moved from cell to cell in TGBp1-expressing *N. benthamiana* leaves but were restricted to single cells in non-transgenic, as well as in TGBp2-, TGBp3-, or TGBp2/TGBp3-expressing transgenic *N. benthamiana* leaves (Lough et al., 1998). In a second study, a PVX construct expressing GUS (PVX.GUS) was injected into *N. clevelandii* trichome cells along with 4.4-kDa, 10-kDa, or 20-kDa fluorescein isothiocynate-conjugated dextrans (F-dextrans) (Angell et al., 1996; Angell and Baulcombe, 1995). The 10-kDa, but not the 20-kDa, F-dextrans moved between adjacent cells. A mutant virus, which had a large segment of TGBp1 deleted, was injected into cells along with F-dextrans, and the 4.4-kDa but not 10-kDa F-dextrans moved from cell to cell (Angell et al., 1996). Thus, TGBp1 induced changes in PD SEL in *N. benthamiana* and *N. clevelandii* (Angell et al., 1996; Lough et al., 1998). In a third study, F-dextrans were injected into cells in PVX CP-expressing transgenic *N. tabacum* leaves. The PVX CP did not increase PD SEL to allow movement of 10-kDa F-dextrans (Oparka et al., 1996).

The combined results of microinjection and biolistic delivery suggest that TGBp1 is likely to be the primary PVX factor that can induce PD gating and can move through PD. Furthermore, the results of biolistic bombardment studies indicate that the PVX TGB and CP cell-to-cell movement are host dependent (Krishnamurthy et al., 2002). The ability of GFP:TGBp2, and GFP:TGBp3 fusion proteins to move from cell to cell in *N. benthamiana* but not in other hosts suggests that *N. benthamiana* has unique properties to promote protein movement (Krishnamurthy et al., 2002).

To further characterize cell-to-cell movement and PD gating activities of PVX TGBp1 and CP, experiments were undertaken in this study to determine if TGBp1 or CP has the ability to move from cell to cell in the PVX hosts *L. esculentum* and *N. clevelandii*, as well as in *N. benthamiana* and *N. tabacum*. In addition, TGBp1-expressing transgenic *N. tabacum* (Verchot et al., 1998) were used in microinjection studies to determine if TGBp1 induces changes in PD SEL in *N. tabacum*. Since *N. tabacum* is often used as an experimental host, we undertook this study to determine whether TGBp1 induces PD gating in this host as well as in *N. benthamiana* or *N. clevelandii* (Lough et al., 1998; Angell et al. 1996).

MATERIALS AND METHODS

Plasmids for bombardment

In this study, green fluorescent protein (GFP) alone, or fused to the 5' end of TGBp1 or CP, was expressed in pRTL2 plasmids. These constructs were developed previously (Yang et al. 2000). pRTL2 contains the CaMV 35S promoter with a dual enhancer, a tobacco etch virus (TEV) translational enhancer at the 5' end, and a CaMV 35S transcription terminator at the 3' end (Yang et al. 2000; Krishnamurthy et al. 2002).

Plant material

N. benthamiana, N. tabacum (cvs. Petit Havana or Samsun nn), *N. clevelandii,* and *L. esculentum* (cv. Trust) leaves were used in these experiments. TGB100 transgenic *N. tabacum* plants expresses PVX TGBp1, are susceptible to PVX infection, and complement cell-to-cell movement of TGBp1-defective PVX viruses, as previously described (Verchot et al., 1995; Verchot et al., 1998).

CF dye test, biolistic bombardment, and epifluorescence microscopy

Source and sink leaves were identified by applying CF dye (Sigma, St. Louis, MO) to the petiole of the most mature leaf of an *N. benthamiana* or *N. tabacum* plant (Krishnamurthy et al., 2002; Oparka et al., 1994; Roberts et al., 1997; Yang et al., 2000). Plants were kept in the dark overnight and the next day leaves were detached and observed using an epifluorescence microscope. Carboxyfluorecein (CF) dye was distributed uniformly in sink leaves. CF dye was detected only in the veins in source leaves.

Following the CF dye experiments, source or sink leaves were detached from plants of similar age and bombarded with plasmids using the PDS 1000/He System (Biorad, Hercules, CA), as described previously (Krishnamurthy et al., 2003; Krishnamurthy et al., 2002; Yang et al., 2000). Leaves were bombarded with 10-20 µg plasmids mixed with 1 mg of 1 µm gold particles. Ten µl of a DNA/gold mixture was loaded on a carrier disk and bombarded to detached leaves as described previously (Yang et al., 2000).

Leaves were observed using a Nikon E600 (Nikon Inc., Dallas, TX) epifluorescence microscope with a Nikon B2A filter cube (containing a 470-490 nm

excitation filter, a DM505 dichroic mirror, and a BA520 barrier filter). Images were captured using the Optronics Magnafire camera (Intelligent Imaging Innovations, Inc., Denver, CO) attached to the Nikon E600 microscope (Krishnamurthy et al., 2002).

Microinjection of mesophyll cells

Microinjection experiments were performed using previously described protocols (Ding et al., 1995; Wolf et al., 1989). Non-transgenic and TGBp1-expressing source tobacco leaves were detached and floated on 0.1 M mannitol in a petri plate. The epidermis was peeled from the abaxial side of the leaf to expose the underlying mesophyll cells. The leaf was placed on wet filter paper under the microscope with the abaxial side up. A 1mM solution of LYCH, and a 5 mM solution of either 4.4-kDa or 9.4-kDa F-dextrans (Sigma, St. Louis, MO) were each prepared in 5mM $KHCO_3$ (pH 8.0), filtered through a 0.5 μm-pore syringe filter, and stored at $4^{\circ}C$ (Fujiwara et al., 1993; Ding et al., 1992; Wolf et al., 1989). Mesophyll cells were injected with LYCH or FITC-dextrans using a PV820 Pneumatic Pico Pump (World Precision Instruments, Sarasota, FL) and an epifluorescence Nikon E600 microscope. Injections were carried out at an eject pressure of 5 to 15 psi. The PV820 has a regulated hold pressure that maintains a constant low pressure following each injection to help maintain cell turgor. The needle was withdrawn slowly to allow the injection wound to seal. The spread of fluorescence from the injected cells to adjacent cells was monitored following injection and images were taken with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) attached to the microscope.

Statistical analyses

All data analysis was conducted using PC SAS Version 8.2 (SAS Institute, Cary, NC) and a 0.05 significance level was used to compare all proportions. Fisher's Exact Tests with PROC FREQ in SAS were used to compare proportions reported in Table 1. Fisher's Exact Tests were used because many of the proportions are small, which will violate a sample size requirement needed to perform chi square tests. Comparisons of plant species for a given plasmid were made, as well as comparisons of plasmid for a given plant species. For overall comparisons that proved significant, pair wise comparisons of the levels were performed. Results of the multiple comparisons in Table 1 are presented with lower case letters (comparing plant species at a given plasmid) and upper case letters (comparing plasmid types at a given plant species) (Krishnamurthy et al., 2003; Krishnamurthy et al., 2002).

RESULTS

GFP:TGBp1 but not GFP:CP moves from cell to cell in four PVX hosts

To determine if cell-to-cell movement is an intrinsic property of TGBp1 or CP, plasmids expressing GFP fused to either TGBp1 or CP were each bombarded to *N. benthamiana*, *N. tabacum*, *N. clevelandii*, and *L. esculentum* leaves. To ensure that leaves used in this experiment were physiologically similar, leaves were identified as source or sink using the carboxyfluorescein (CF) dye test. Source leaves were taken from similar, untreated plants for biolistic bombardment experiments. A plasmid expressing only GFP was used as a control, and movement of fluorescence was monitored one-day post bombardment (1dpb). These plasmids were used in previous studies exploring cell-to-cell movement of GFP:TGBp1 or GFP:CP (Krishnamurthy et al., 2002; Yang et al.,

2000).   Fluorescence would be observed in clusters of adjacent cells or in single cells if GFP:TGBp1 and GFP:CP have the ability to move from cell to cell or are restricted to single cells, respectively.

Fluorescence was detected primarily in single epidermal cells in source leaves bombarded with plasmids expressing GFP alone (Fig. 1).   GFP was detected in two or more adjacent cells on rare occasions (Table 1).   Occasions of GFP fluorescence in adjacent cells were reported in previous studies and might be due to simultaneous expression of GFP in neighboring cells (Itaya et al., 1997; Krishnamurthy et al., 2002; Mitra et al., 2003; Morozov et al., 1997; Yang et al., 2000).

Statistical analyses were conducted to compare the proportions of cell clusters containing GFP, GFP:TGBp1 or GFP:CP in each plant species.   GFP:TGBp1 was observed in multiple-cell (two or more adjacent cells) clusters in *N. tabacum*, *N. clevelandii*, and *N. benthamiana* and *L. esculentum* (Fig. 1)   In each plant species that was tested, the proportions of sites containing GFP:TGBp1 in multiple-cell clusters were significantly greater than the proportion of sites that were cell clusters containing GFP ($P<0.05$).   In each *Nicotiana* species, between 21% and 34% of sites viewed were clusters of two to five adjacent cells (Table 1).   In *L. esculentum*, approximately 14% of sites were multiple-cell clusters (two to five adjacent cells), indicating that GFP:TGBp1 movement in tomato is less frequent than in tobacco species (Table 1).

In *N. benthamiana* leaves, the proportions of sites containing GFP:CP in multiple-cell clusters was significantly greater than the proportion of sites containing GFP in cell clusters (Table 1; $P<0.05$).   Twenty-two percent of the GFP:CP-containing fluorescent sites viewed in *N. benthamiana* were multiple-cell clusters (Fig. 1 and Table 1). GFP:CP accumulated primarily in single epidermal cells in *N. tabacum*, *N. clevelandii*, and *L.*

esculentum (Table 1). The differences between GFP and GFP:CP accumulation in cell clusters in *N. tabacum, N. clevelandii,* and *L. esculentum* were not significantly different (P>0.05). These data suggest that TGBp1, but not CP, has the activities needed to move between adjacent cells in *N. tabacum, N. benthamiana,* and *L. esculentum*.
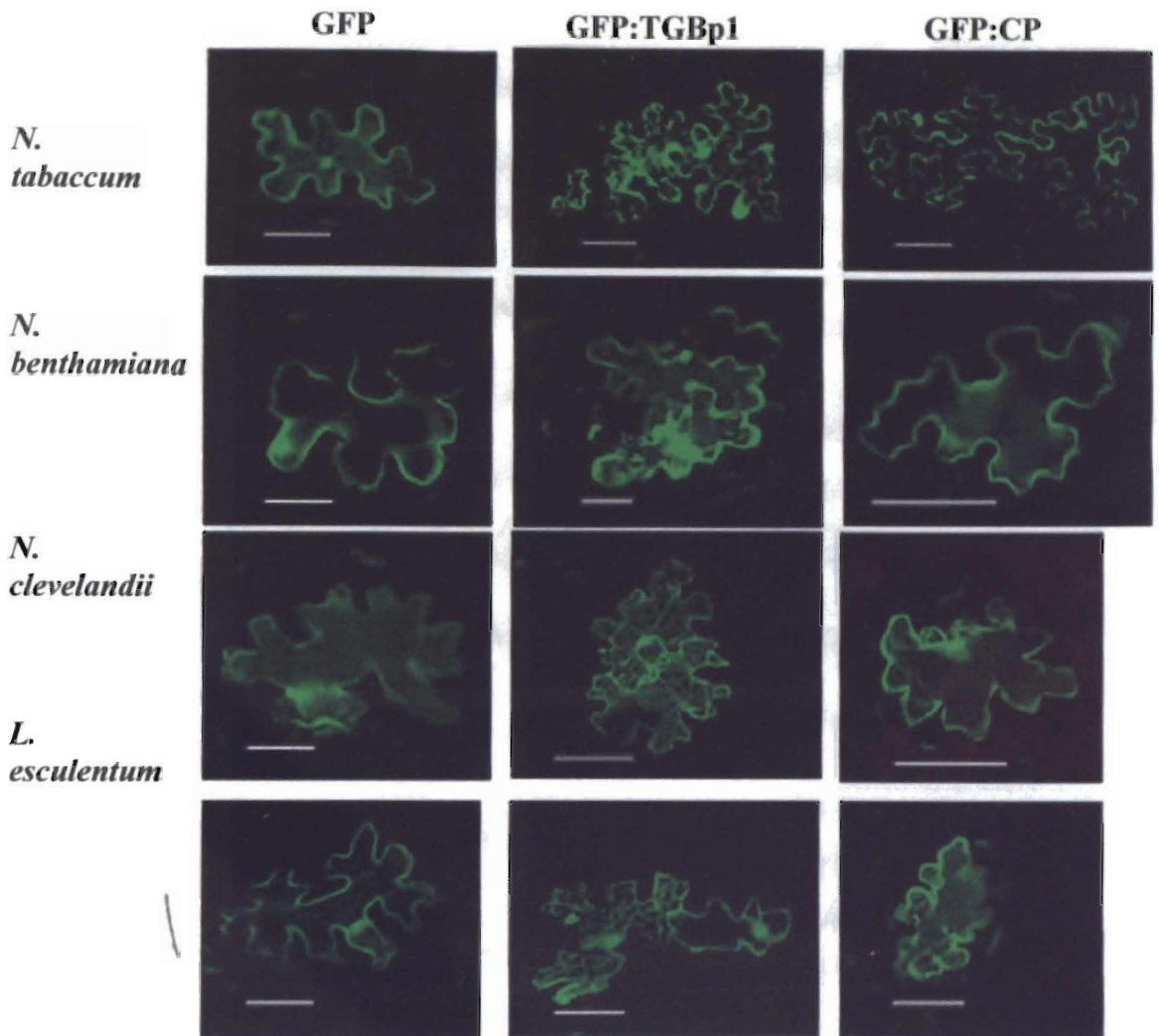
Fig. 1. Images of leaf epidermal cells containing fluorescence due to GFP, GFP:TGBp1, or GFP:CP. Plasmids expressing GFP, GFP:TGBp1, or GFP:CP were biolistically delivered to *N. benthamiana, N. tabacum, N. clevelandii*, or *L. esculentum* leaves. Plant species for each row of images is indicated on the right. Fusion proteins for each column of images are indicated at the top of each column. Images were taken using a 20X objective lens. Scale bars = 20 μm.

When statistical comparisons were made among plant species, GFP performed the same in all plant species (Table 1). There were no significant differences between plant species in the proportions of sites containing GFP in cell clusters (Table 1; P<0.05). However, there was some variation in the frequency of GFP:TGBp1 movement among the different plant species. The proportion of cell clusters in *N. benthamiana* and *L. esculentum* represented extreme values that were significantly different (Table 1; P<0.05). The proportions of cell clusters in *N. tabacum* and *N. clevelandii* fell between the two extreme values and were not significantly different from either *L. esculentum* or *N. benthamiana* (Table 1; P>0.05). The proportions of cell clusters containing GFP:CP in *N. tabacum*, *N. clevelandii*, or *L. esculentum* were not significantly different from each other. However, the proportions of GFP:CP-containing cell clusters in *N. benthamiana* were significantly different from the other three plant species (Table 1; P<0.05). These data correlate with previous data that GFP:CP moves from cell to cell only in this host species (Krishnamurthy et al., 2002).

Table 1. Cell-to-cell movement of GFP, GFP:TGBp1, and GFP:CP

Proportion of sites containing GFP activity in multiple cell clusters[a]

Plants	GFP	GFP:TGBp1[b]	GFP:CP
N. tabacum	3.3%(3/90)aB	22.2%(20/90)abA	2.3%(2/88)bB
N. benthamiana	3.3 (3/90)aC	33.6 (36/107)aA	21.8 (31/142)aB
N. clevelandii	6.8 (6/88)aB	21.0 (20/95)abA	1.1 (1/90)bB
L. esculentum	2.3 (2/88)aB	14.4 (13/90)bA	3.3 (3/90)bB

[a] Percentages of fluorescent cell clusters observed 1 dpb in source leaves are indicated. Multiple cell clusters are defined as two or more adjacent cells showing GFP fluorescnece. The total numbers of fluorescent sites that are multiple cell clusters relative to the total number of fluorescent sites are in parentheses. Values followed by the same lowercase letter within each column are not significantly different using Fisher's Exact Test at P>0.05. Values followed by the same uppercase letter in each row are also not significantly different using Fisher's Exact Test at P>0.05.

[b] In comparing plants bombarded with GFP:TGBp1 plasmids, *N. tabacum* and *N. clevelandii* values of 22.2 and 21.0 are followed by "ab". This means that 22.2 or 21.0 are not significantly different from 33.6, but they are also not significantly different from 14.4. However, 33.6 and 14.4 are significantly different as indicated by the "a" and "b" designations.

Transgenically expressed TGBp1 increases PD SEL

TGB100 transgenic *N. tabacum* (Verchot et al., 1998) expresses the TGBp1 protein and has been used previously to study PVX and protein cell-to-cell movement (Krishnamurthy et al., 2002). TGBp1-deficient PVX viruses cannot move from cell to cell in non-transgenic *N. tabacum* but can move between adjacent cells in TGBp100 transgenic *N. tabacum* (Verchot et al., 1998). Thus, the transgenically expressed TGBp1 protein is functional to promote virus movement (Verchot et al., 1998).

As mentioned in Chapter I, it was determined that the PD SEL in tobacco leaves is below 1-kDa using microinjection (Robards, 1990). The low molecular weight (457 kDa) Lucifer Yellow CH dilithium salt (LYCH) is a fluorescent dye that can pass through PD, while F-dextrans of 4.4- kDa or 9.5-kDa are often restricted to single cells in tobacco leaves (Ding et al., 1992; Wolf et al., 1989). LYCH was injected into non-transgenic and transgenic mesophyll cells, as a control, to ensure PD are not closed as a result of tissue preparation. The 4.4-kDa or 9.5-kDa F-dextrans were microinjected into mesophyll cells of non-transgenic and TGB100 transgenic *N. tabacum* source leaves, to determine if transgenically expressed TGBp1 can increase PD SEL. Intercellular movement of the fluorescently labeled molecules was recorded.

Table 2. Movement of fluorescent LYCH, 4.4 kDa dextrans, and 9.5 kDa dextrans between mesophyll cells in leaves of non-transgenic or TGB100 transgenic *N. tabacum*. [a]

Dye	Non-transgenic	TGB100
LYCH	84% (16/19)	85% (11/13)
4.4 kDa dextrans	29 (4/14)	79 (14/18)
9.5 kDa dextrans	0 (0/11)	69 (11/16)

[a] Percentages of injections resulting in dye movement between adjacent cells is indicated. The proportions of injected cells in which dye moved out into adjacent cells, relative to the total number of injected cells are indicated in parentheses.

Fig. 2 shows representative images collected within three to five minutes after injection of LYCH, 4.4-kDa F-dextrans, or 9.5-kDa F-dextrans in non-transgenic and TGB100 leaves. LYCH moved out of 84 and 85 % of the injected cells in non-transgenic or TGB100 leaves, respectively (Table 2). In non-transgenic leaves, the 4.4-kDa F-dextrans moved out of 29 % of the injected cells whereas the 9.5-kDa F-dextrans did not move out of any injected cells (Table 2). In TGB100 transgenic *N. tabacum*, the percentage of cells allowing movement of 4.4-kDa and 9.5-kDa F-dextrans increased to 79% and 69%, respectively (Table 2). Thus, the transgenically expressed TGBp1 protein increased PD SEL.
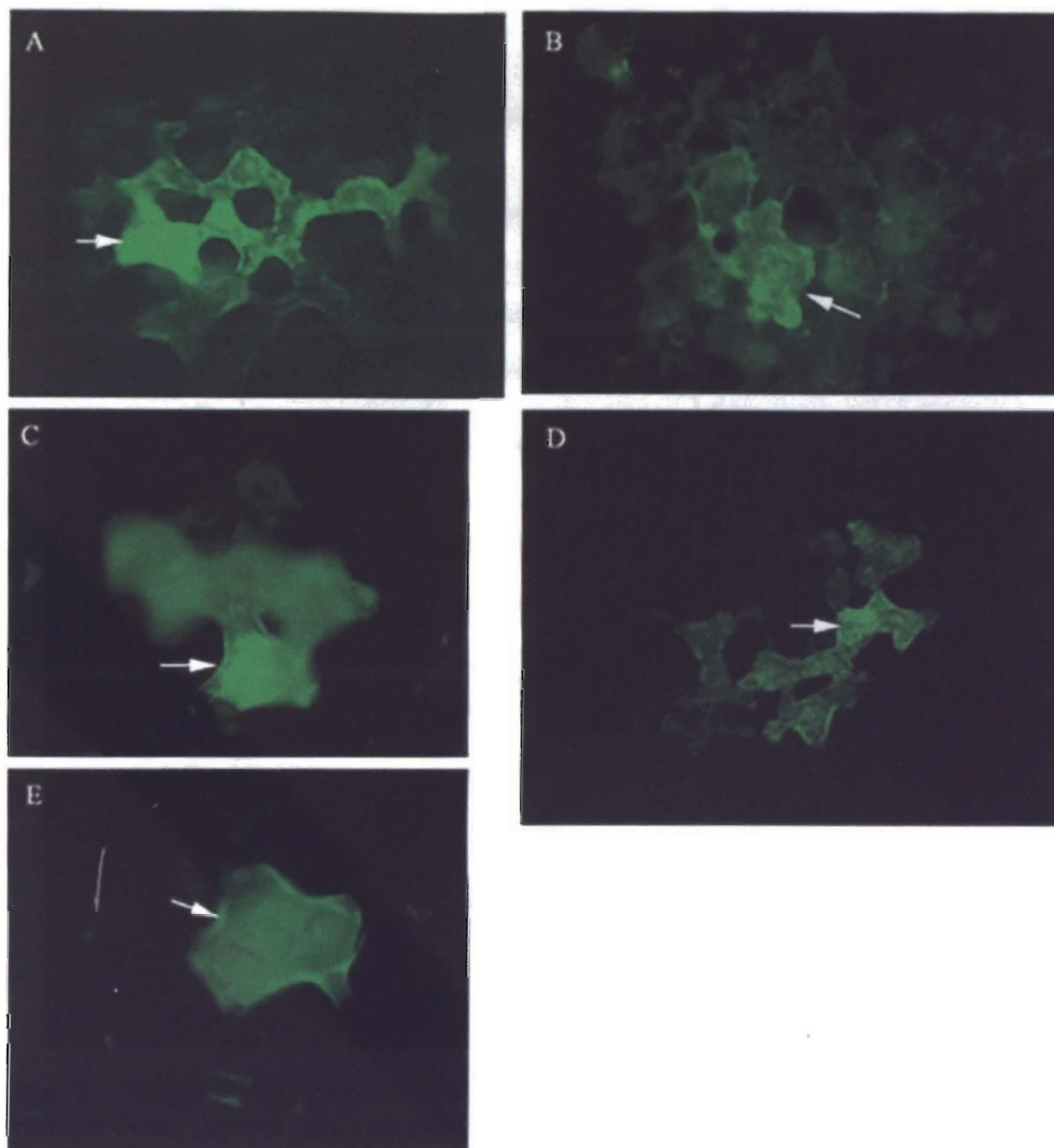
Fig. 2. Images of fluorescence in mesophyll cells following injection of LYCH or F-dextran dyes. Arrowheads indicate dye or dextran injected cell in each panel. (A), (B) show extensive spread of LYCH fluorescence between mesophyll cells in non-transgenic and TGB100 transgenic leaves, respectively. (C), (D), respectively show cell-to-cell movement of 4.4-kDa F-dextrans in non-transgenic leaves and 9.5-kDa F-dextrans in TGB100 leaves. (E) 9.5-kDa F-dextrans restricted to single mesophyll cell in non-transgenic.

DISCUSSION

The PVX TGBp1 protein is the only viral protein that is frequently detected in multiple-cell clusters in the four PVX hosts used in this study. Combining the results of this and previous studies, we have demonstrated that GFP:TGBp2, GFP:TGBp3, and GFP:CP accumulate frequently in multiple cell clusters in *N. benthamiana* leaves, but occur predominantly in single cells in *N. tabacum, N. clevelandii,* and *L. esculentum* leaves (Krishnamurthy et al., 2003; Krishnamurthy et al., 2002). Taken together, these data indicate that TGBp1, unlike the other PVX proteins, is a factor that moves intercellularly in several PVX hosts.

The results of GFP:CP and GFP:TGBp1 analyses (Table 1) indicate that there are statistical differences in the numbers of multiple cell clusters detected among the different plant species. In the case of GFP:TGBp1, differences resided in the proportions of multiple cell clusters viewed in leaves of different plant species. The proportions of cell clusters due to GFP:TGBp1 in *L. esculentum* and *N. benthamiana* were significantly different, while the proportions of cell clusters in *N. tabacum* and *N. clevelandii* were between these two extremes. GFP:CP accumulated with some frequency in cell clusters in *N. benthamiana* leaves but was restricted primarily to single epidermal cells in other plant species tested. Evidence that PVX TGBp2, TGBp3, or CP can move from cell to cell in *N. benthamiana* but not in other plant species supports the argument that protein movement is enhanced in *N. benthamiana* relative to other plant species (Krishnamurthy et al., 2002; Krishnamurthy et al., 2003). The statistical differences between the results in *N. benthamiana* and *L. esculentum* leaves expressing either GFP:TGBp1 or GFP:CP also indicate that there are possible differences between plant species (Table 1). Plant species may affect protein movement due to differences in the following: the regulatory

32

mechanism of macromolecular transport through PD, the mechanism of the induction of PD gating, the extent to which the PD SEL may be altered, or the cellular factors contributing to PD transport.

The results presented in this and in previous studies (Krishnamurthy et al., 2003; Krishnamurthy et al., 2002) suggest that movement of the PVX TGB and CP proteins in *N. benthamiana* might reflect unique properties of this host. It is possible that *N. benthamiana* either contains a positive factor that promotes protein movement through PD, or lacks a repressor which functions in other plant species to limit protein trafficking through PD. Data obtained from studies using *N. benthamiana* have been used to build models explaining the role of the potexvirus TGB proteins in plasmodesmata transport. These models likely reflect unique properties of *N. benthamiana* plants and might not explain viral protein functions in a broader context.

Experiments were conducted using source leaves. Source leaves were used because previous studies of *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), and PVX have shown that PD gating activities and protein cell-to-cell movement often varies between young and mature leaves (Ding et al., 1992; Itaya et al., 1998; Krishnamurthy et al., 2002). For example, in microinjection studies, transgenically expressed TMV movement protein modified PD SEL in mature source leaves but not in young sink leaves (Ding et al., 1992). In biolistic bombardment experiments using plasmids expressing GFP fused to the CMV 3a movement protein (3aMP:GFP), CMV 3a MP:GFP moved between adjacent cells in mature source leaves but not in young sink leaves (Itaya et al., 1998). GFP:TGBp1 movement has been observed in both source and sink *N. tabacum* leaves (unpublished data), however GFP:TGBp2 and GFP:TGBp3

moved from cell to cell in TGB100 source but not sink leaves (Krishnamurthy et al., 2002).

Another reason sink leaves were not used in this study is because of reports indicating nonspecific protein movement in sink leaves of *N. tabacum* and *N. benthamiana* (Imlau et al., 1999; Krishnamurthy et al., 2002; Oparka et al., 1999; Itaya et al., 2000; Crawford and Zambryski, 2001). In sink leaves the PD are physiologically different in some manner than in fully expanded, mature leaves. Simple, single-channeled PD are more common in young leaves while branched secondary PD are more frequent in mature fully expanded source leaves (Itaya et al., 1998; Oparka et al., 1999). Nonspecific movement of proteins through simple, non-branched PD in young sink leaves may reflect important differences in PD permeability at the different leaf developmental stages (Oparka et al., 1999). In young leaves there may be a greater need for intercellular trafficking of macromolecules important for leaf development (Itaya et al., 2000; Oparka et al., 1999). As the leaves mature nonspecific movement is down regulated, and PD transport of macromolecules in mature leaves depends on a mechanism that regulates intercellular transport of macromolecules (Itaya et al., 2000; Oparka et al., 1999).

The results of the microinjection experiments (Table 2 and Fig. 2) demonstrate that the transgenically expressed TGBp1 promoted PD transport of 4.4-kDa and 9.5-kDa F-dextrans. These data suggest that TGBp1 increases plasmodesmal SEL in *N. tabacum* in a manner that is similar to reports in *N. clevelandii* and *N. benthamiana* (Angell et al., 1996; Lough et al., 1998). Movement of 10-kDa or 20-kDa F-dextrans from the injected mesophyll cell into adjacent cells was observed in transgenic *N. benthamiana*, expressing the WClMV TGBp1 protein (Lough et al., 1998). This was determined to be an

34

indication that the transgenically expressed WClMV TGBp1 increased PD SEL (Lough et al., 1998). The effects of PVX TGBp1 protein on PD in *N. clevelandii* trichome cells was also studied by co injecting F-dextrans and either a PVX virus expressing GUS (PVX.GUS) or a mutant PVX.GUS lacking a large segment of the TGBp1 open reading frame (Angell et al., 1996). Large 10-kDa and 20-kDa F-dextrans could move from the injected cell into adjacent cells in the presence of PVX.GUS but could not move from cells co-injected with the mutant PVX.GUS (Angell et al., 1996). Thus, deletion of TGBp1 from the PVX genome eliminated the capacity of PVX to increase the PD SEL to allow movement of 10-kDa or 20-kDa F-dextrans.

The ability of the potexvirus TGBp1 to increase the PD SEL in *N. tabacum* supports previous studies indicating TGBp1 is a factor that induces PD gating for virus movement in *N. benthamiana* and *N. clevelandii* (Angell et al., 1996; Lough et al., 1998). Taken together these data indicate TGBp1 has the unique ability to move from cell to cell in four PVX hosts and induces PD gating in three PVX hosts. Furthermore, it has been shown that TGBp1 promotes cell-to-cell movement of TGBp2 and TGBp3 in *N. tabacum* leaves (Krishnamurthy et al., 2002). When plasmids expressing GFP fused to TGBp2 or TGBp3 were bombarded to non-transgenic or TGB100 transgenic *N. tabacum* leaves (Krishnamurthy et al., 2002), GFP:TGBp2 and GFP:TGBp3 each accumulated in single cells in non-transgenic leaves but were able to move from cell to cell in transgenic leaves (Krishnamurthy et al., 2002). Thus, TGBp1 likely functions as the primary factor for virus and protein cell-to-cell movement.

CHAPTER III

MUTATIONAL ANALYSIS OF TGBp1 INDICATES CELL-TO-CELL MOVEMENT OF TGBp1 MIGHT NOT BE ESSENTIAL FOR VIRUS CELL-TO-CELL MOVEMENT

ABSTRACT

The TGBp1 protein of *Potato virus X* (PVX) has ATPase activity, interacts with PVX TGBp2, TGBp3, CP, induces plasmodesmata (PD) gating, moves through PD, is a suppressor of plant gene silencing, and is required for virus cell-to-cell movement. Ten insertion and two substitution mutations were introduced into TGBp1 coding region of PVX to identify sequences required for virus cell-to-cell movement. Plasmids expressing GFP fused to wild-type and mutant TGBp1 proteins were also prepared and were biolistically bombarded to tobacco leaves to study protein cell-to-cell movement. While TGBp1 ATPase activity is important for virus cell-to-cell movement, data in this study suggests protein cell-to-cell movement might not be essential for virus cell-to-cell movement. Mutations in TGBp1 affecting interactions with TGBp2 and TGBp3, but not CP, were identified. Evidence indicated that complexes containing TGBp1, TGBp2, and TGBp3 might be important for virus cell-to-cell movement.

INTRODUCTION

Potato virus X (PVX) requires all three highly conserved triple gene block proteins, TGBp1, TGBp2, and TGBp3, to move cell-to-cell (Lough et al., 1998; Lough et al., 2000; Memelink et al., 1990; Morozov et al., 1987; Skryabin et al., 1988; Yang et al., 2000). Potexvirus and hordeivirus TGBp1 proteins can induce PD gating, move cell-to-

cell, bind RNA, and have ATPase activity (Donald et al., 1997; Rouleau et al., 1994; Yang et al., 2000). The potexvirus TGBp1 might transport viral RNAs through PD into adjacent cells, similar to the *Tobacco mosaic virus* (TMV) P30 movement protein (MP) (Donald et al., 1997; Lough et al., 2000; Lough et al., 1998). Cell-to-cell movement of TGBp1 is restricted in transgenic tobacco expressing either the PVX CP or the combined TGBp2 and TGBp3 (Krishnamurthy et al., 2002). Thus, TGBp1 might interact either with CP, or with a complex of TGBp2 and TGBp3 proteins, in a manner that regulates TGBp1 cell-to-cell movement (Krishnamurthy et al., 2002).

In this study a series of insertion and substitution mutations were introduced into the TGBp1 coding sequence of PVX and in plasmids containing the green fluorescent protein (GFP) gene fused to TGBp1. Experiments were conducted to identify amino acid sequence domains within TGBp1 contributing to virus cell-to-cell and systemic movement, protein cell-to-cell movement, and interactions with TGBp2, TGBp3, and CP. In this study mutations in TGBp1 were identified that: a) have different effects on virus and protein movement in *Nicotiana benthamiana* and *N. tabacum*; b) alter the TGBp1 ATPase active sites inhibiting protein and virus movement in both *Nicotiana* species; or, c) alter interactions with TGBp2 and TGBp3.

MATERIALS AND METHODS

Bacterial strains and plasmids

All plasmids used in this study were used to transform *Escherichia coli* strain JM109. The pPVX204 plasmid (Fig. 1A) was prepared previously and contains cDNA of the PVX genome adjacent to the *Cauliflower mosaic virus* (CaMV) 35S promoter. The green fluorescent protein (GFP) gene was inserted into the genome under a duplicated

subgenomic promoter (Verchot et al., 1995). GFP is used as a visual marker to study the spread PVX infection (Baulcombe et al., 1995). The pPVX204.Δ25K plasmid (Fig. 1B) was also prepared previously (Yang et al., 2000). The pPVX204.Δ25K plasmid has 357 nts deleted from the TGBp1 gene. This mutation inhibits cell-to-cell movement of PVX (Verchot et al. 1998; Yang et al., 2000).

The QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) was used to introduce twelve separate mutations (m1 through m9, m11 through m13) into the TGBp1 gene in pPVX204 plasmids. Ten insertion mutations (m1 through m9, and m11) were introduced at regular intervals throughout the TGBp1 coding region. The QuikChange Site-Directed Mutagenesis kit uses two mutagenesis oligonucleotides in the forward and reverse orientation (Table 1). Mutations m1 though m9 and m11 were generated using oligonucleotides that contained nine nt insertion mutations, each encoding a *Bam*HI restriction site and the tripeptide A-G-S (Table 1 and Fig. 2). Two substitution mutations (m12 and m13) were also generated replacing sequences encoding two ATPase active site motifs G-K-S and D-E-Y with nine nts encoding A-E- S and R-R-F, respectively (Table 1 and Fig. 2). The G-K-S site binds ATP and the D-E-Y motif binds Mg^{2+} both of which are necessary for TGBp1 ATPase activity (Morozov et al., 1999).

For site-directed mutagenesis, a fragment of pPVX204 was PCR amplified using a forward primer that anneals to the 3' end of the replicase gene (GCC AAA CAC CAC TGC ATA CCA GAG GAA ATC), as well as a reverse primer (GGC GGT CGA CAT TTA CTT GTA CAG CTC GTC CAT) that overlaps the GFP gene and contains a *Sal*I (underlined) site. The PCR products were ligated into pGEM-T Easy vector (Promega, Madison, WI). Then mutations were separately introduced into the pGEM-T clone using the forward and reverse

primers indicated in Table 1. The successful generation of mutant constructs were confirmed by restriction digestion and sequencing.

To introduce mutations into the PVX genome, pGEM-T plasmids encoding mutations m1 through m8 and PVX204 plasmids were digested with *Avr*II and *Apa*I. In addition, pGEM-T plasmids encoding mutations m9 through m11 and PVX204 were digested with *Apa*I and *Sal*I. Digested fragments containing the mutations and digested pPVX204 plasmids were ligated together. The resulting mutant pPVX204 constructs are shown in Fig. 1A.
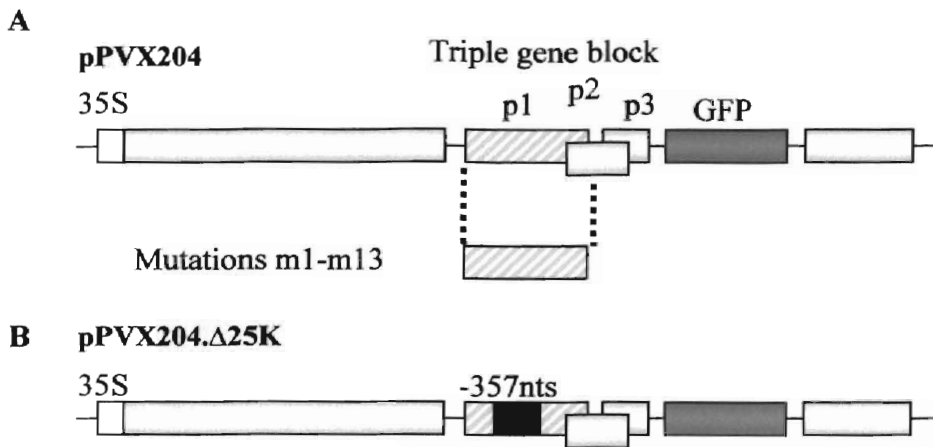
Fig. 1. Schematic depicting plasmids containing cDNA of the PVX genome (open boxes) under the CaMV 35S promoter. Open boxes at the 5' end of the PVX genomes represent the CaMV 35S promoter, the light gray boxes represent PVX open reading frames, the dark gray boxes represent GFP, and the hatched boxes represent and wild-type or mutant TGBp1. **(A)** pPVX204 contains cDNA of the PVX genome fused to the CaMV 35S promoter. Mutations m1 through m13 were inserted into the TGBp1 coding sequence of pPVX204 by site directed mutagenesis. (B) The plasmid pPVX204.Δ25K has 357 nts deleted within the TGBp1 gene (Verchot et al., 1998; Yang et al., 2000).

Table 1. TGBp1 forward and reverse mutagenesis primers

M1	Forward: AGGACTTCCAAAGCCGGATCCTCTTTAGATTCA
	Reverse: TGAATCTAAAGAGGATCCGGCTTTGGAAGTCCT
M2	Forward: AAGTCCACAGCCGCCGGATCCCTAAGGAAGTTG
	Reverse: CAACTTCCTTAGGGATCCGGCGGCTGTGGACTT
M3	Forward: GGTGTCCCTGACGCCGGATCCAAGGTGAGTATC
	Reverse: GATACTCACCTTGGATCCGGCGTCAGGGACACC
M4	Forward: CCTGAGGGCAACGCCGGATCCTTCGCAATCCTC
	Reverse: GAGGATTGCGAAGGATCCGGCGTTGCCCTCAGG
M5	Forward: AACTCATACCAGGCCGGATCCGCACTTTTTGCT
	Reverse: AGCAAAAAGTGCGGATCCGGCCTGGTATGAGTT
M6	Forward: CCCCACTTCTACGCCGGATCCTTGGAAACATCA
	Reverse: TGATGTTTCCAAGGATCCGGCGTAGAAGTGGGG
M7	Forward: GGCTGTGGCTTCGCCGGATCCGATTTCGAGACC
	Reverse: GGTCTCGAAATCGGATCTGGCGAAGCCACAGC
M8	Forward: ACTGGCATATTCGCCGGATCCAAAGGGCCCCTA
	Reverse: TAGGGGCCCTTTGGATCCGGCGAATATGCCAGT
M9	Forward: ACAACACTGTCCGCCGGATCCAGGCATGGTGGT
	Reverse: AACACCATGCCTGGACCGGCGGACAGTGTTGT
M11	Forward: ACAGCTTTCTACGCCGGATCCAACGCTATCACC
	Reverse: GGTGATAGCGTTGGATCCGGCGTAGAAAGCTGT
M12	Forward: GTAGCCGGAGCCGCGGAAACCACAGCCCTAAGG
	Reverse: CCTTAGGGCTGTGGTTTCGCGGCTCCGGCTAC
M13	Forward: TTCGCAATCCTCAGGAGATTCACTTTGGACAAC
	Reverse: GTTGTCCAAAGTGAATCTCCTGAGGATTGCGAA

Primers used for site directed mutagenesis kit were used to introduce mutations into pPVX204 and pRTL2-GFP:TGBp1 plasmids GFP:TGBp1 plasmids. Mutations m1 through m11 are nine nt insertion mutations that encode *Bam*HI restriction sites. The mutations are underlined. Mutations m12 and m13 are substitution mutations, are also underlined, and are highlighted in gray.

1

MDILISSLKSLGYSRTSK**AGS**SLDSGP<u>LVVHAVAG</u>

   12   **2**   **3**

A**AES**TA**AGS**LRKLILRHPTFT<u>VHTLGVPD</u>**AGS**KVS

                       4   **13**

<u>IRTRGI</u>QKPGPIPEGN**AGS**<u>FAIL</u>**RRF**TLDNTTRNSY

   5   **6**

Q**AGS**<u>ALFADPYQAPEFSLEPHFY</u>**AGS**LETSFRVPR

               7   **8**

KVADLIAGCGF**AGS**DFETNSQEEGHLEITGIF**AGS**

                       9

KGPLLGKVIAIDEESETTLS**AGS**RHG<u>VEFVKPCQV</u>

                                     11

<u>TGLELKVVAGSTIVSAAPIEEIGQSTAFY</u>**AGS**NAIT

<u>RSKGL</u>TYVRAGT

Fig. 2. Mutations introduced into the PVX TGBp1 amino acid sequence. Conserved helicase motifs I, IA through IV are underlined (Morozov et al., 1999). The TGBp1 amino acid sequence is in gray, and the mutations are numbered and highlighted in black. Gray boxes surround the two substitution mutations (m12 and m13).

Plasmids pRTL2-GFP and pRTL2-GFP:TGBp1, prepared previously, were used to study cell-to-cell movement of TGBp1 (Yang et al. 2000; Krishnamurthy et al. 2002). Mutations m1 through m13 were also introduced into pRTL2-GFP:TGBp1 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the forward and reverse primers listed in Table 1.

Plant material

Non-transgenic *N. benthamiana*, non-transgenic *N. tabacum* (cvs. Petit Havana), and transgenic *N. tabacum* expressing either the TGBp1 gene (line TGB100), the combined TGBp2 and TGBp3 genes (line TGB600), or CP genes (line CP) were used in these experiments. TGB100, TGB600, and CP transgenic *N. tabacum* plants are susceptible to PVX infection, and the transgenically expressed proteins were shown to complement cell-to-cell movement of movement-defective PVX viruses (Verchot et al., 1995; Spillane et al., 1997; Verchot et al., 1998). Plants were grown either in a growth chamber at 25°C with 14 h light and 10 h dark or under greenhouse conditions with 14 h daylight supplemented with growth lights and 10 h dark.

CF dye test, biolistic bombardment, and plant inoculations

Leaf developmental stage was determined for studies of protein cell-to-cell movement. Source and sink leaves were identified by applying carboxyfluorescein (CF) dye (Sigma, St. Louis, MO) to the petiole of the most mature leaf of an *N. benthamiana* or *N. tabacum* plant (Krishnamurthy et al., 2002; Oparka et al., 1994; Roberts et al., 1997; Yang et al., 2000). Plants were kept in the dark overnight and the next day leaves were detached and observed using an epifluorescence microscope. CF dye was detected uniformly in sink leaves. CF dye was detected only in the veins in source leaves.

43

Following the CF dye test, source or sink leaves were detached from plants of similar age and bombarded with plasmids using the PDS 1000/He System (Biorad, Hercules, CA), as described previously (Krishnamurthy et al., 2003; Krishnamurthy et al., 2002; Yang et al., 2000). Leaves were bombarded with 10-20 µg pRTL2-GFP, pRTL2-GFP:TGBp1, or mutant pRTL2-GFP:TGBp1 plasmids mixed with 1 mg of 1 µm gold particles. Ten µl of a DNA/gold mixture was loaded on a carrier disk and bombarded to detached leaves as described previously (Yang et al., 2000).

To test the effects of the TGBp1 mutations on virus cell-to-cell and systemic movement wild-type and mutant pPVX204 plasmids were used to inoculate *N. benthamiana* and *N. tabacum* plants. Each leaf was rub-inoculated with carborundum and 20 µg of plasmid. Two leaves per plant were inoculated.

Visualization of GFP

Cell-to-cell movement of PVX204 viruses and GFP fusion proteins was monitored using a Nikon E600 (Nikon Inc., Dallas, TX) epifluorescence microscope with a B2A filter cube (containing a 470-490 nm excitation filter, a DM505 dichroic mirror, and a BA520 barrier filter). Images were captured using the Optronics Magnafire camera (Intelligent Imaging Innovations, Inc., Denver, CO) attached to the Nikon E600 microscope (Krishnamurthy et al., 2002).

Plants inoculated with PVX204 viruses were also monitored at 7, 14, and 21 days post inoculation (dpi) using a hand-held model B-100 BLAK-RAY long wave ultraviolet (UV) lamp (Ultraviolet Products, Upland, CA). The data was recorded with a Sony Digital Still Camera model DSC-F717 (Sony Corporation of America, New York City, New York). All images taken with the Optronics or Sony cameras were edited using Adobe Photoshop version 4.0 software (Adobe Systems Inc., San Jose, CA).

44

Statistical analyses

Dr. Mark Payton (Department of Statistics, OSU) provided statistical analyses of the data in each table. Statistical analyses were conducted using PC SAS Version 8.2 (SAS Institute, Cary, NC) and a 0.05 significance level was used to compare all proportions. Fisher's Exact Tests with PROC FREQ in SAS were used to compare proportions reported in Tables 4 through 7. Fisher's Exact Tests were used because many of the proportions are small, which will violate a sample size requirement needed to perform chi square tests. Pairwise comparisons among the data obtained from one plant system are presented with lower case letters.

RESULTS

TGBp1 mutations affected virus cell-to-cell and systemic movement

Again, the pPVX204 plasmid encodes the PVX genome adjacent to the CaMV 35S promoter and contains the GFP gene inserted into the viral genome (Fig. 1A). The pPVX204 plasmids can be rub-inoculated to tobacco leaves and GFP expression is used to monitor the systemic spread of PVX infection. To study the role of the PVX TGBp1 protein in virus cell-to-cell movement mutations were introduced into pPVX204 plasmids. Ten insertion mutations, encoding A-G-S, (named m1 through m9 and m11) were introduced at regular 18 amino acid intervals throughout the TGBp1 coding region (Fig. 2). In addition two substitution mutations (m12 and m13) were introduced replacing sequences encoding the G-K-S and D-E-Y ATPase active site motifs with sequences encoding A-E-S and R-R-F, respectively (Fig. 2).

Wild-type PVX204 and mutant viruses were inoculated to *N. benthamiana* and *N. tabacum* leaves. Using a UV lamp, GFP fluorescence was detected in wild-type PVX204

inoculated leaves at approximately 7 dpi (Fig. 3B and 3E). Fluorescence was evident in the upper leaves of *N. tabacum* and *N. benthamiana* plants by approximately 14 dpi. As a negative control, plants were inoculated with PVX204.Δ25 virus. PVX204.Δ25 has 357 nts deleted within the TGBp1 coding sequence and was shown previously to be defective in virus cell-to-cell movement (Verchot et al., 1998; Yang et al., 2000). PVX204.Δ25 did not move from cell to cell or systemically in these plants (Fig. 3A and 3D).

PVX204.TGBp1m3 moved from cell to cell in *N. benthamiana* plants but was unable to move from cell to cell in *N. tabacum* plants (Fig. 3G and 3J; Table 2). PVX204.TGBp1m3 systemic infection was delayed in comparison to wild-type PVX204. While GFP fluorescence and mosaic symptoms due to PVX204 was detected in upper leaves between 7 and 10 dpi in *N. benthamiana*, GFP fluorescence due to PVX204.TGBp1m3 was evident in upper non-inoculated leaves by 21 dpi. PVX204.TGBp1m4 moved from cell to cell and systemically in both *N. benthamiana* and *N. tabacum* plants (Fig. 3C, 3F, and Table 2). Systemic infection due to PVX204.TGBp1m4 was observed in *N. benthamiana* and *N. tabacum* leaves by 10 dpi. None of the other mutant viruses moved from cell to cell or systemically in inoculated plants.
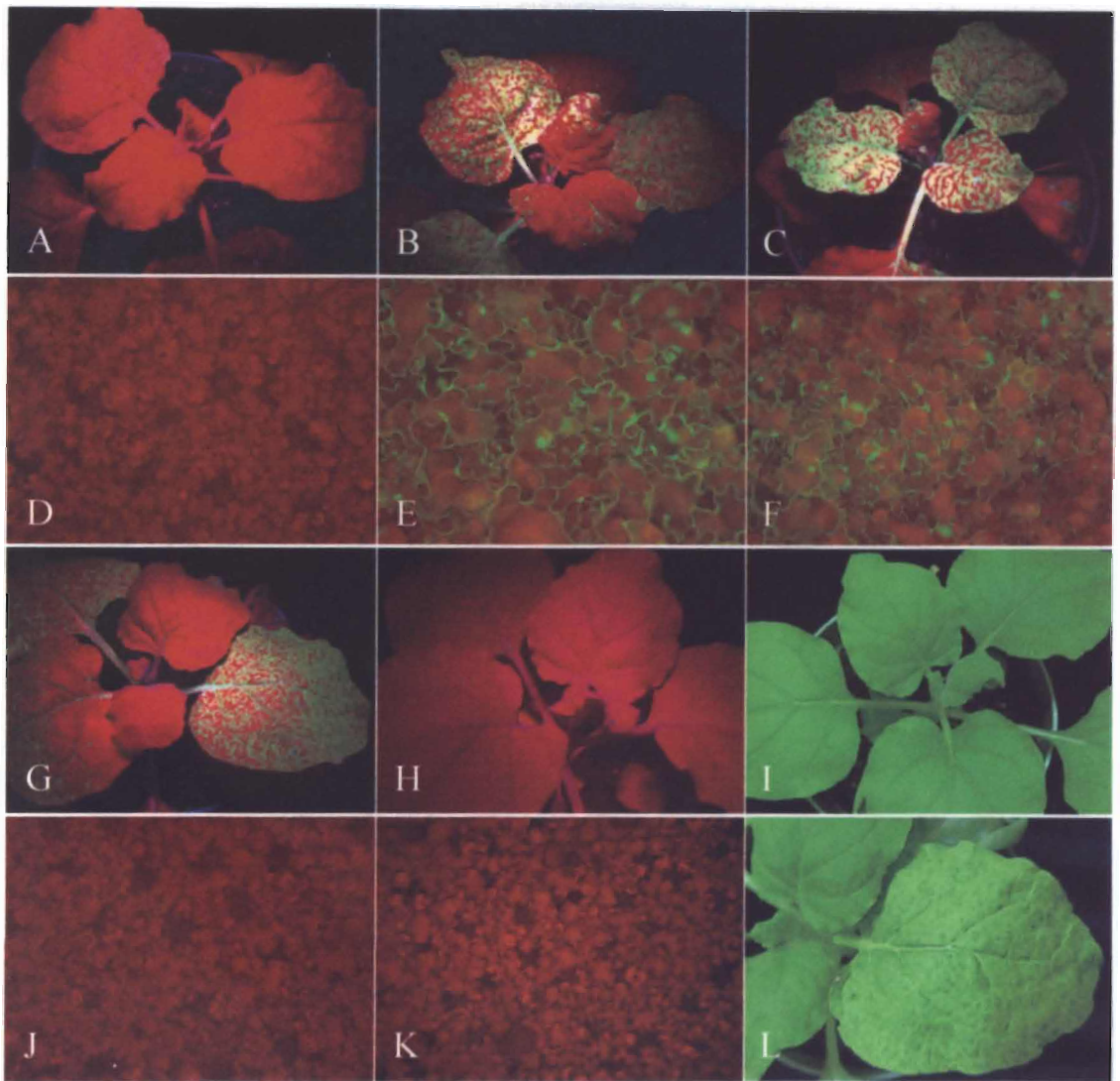
Fig. 3. Images taken at 21 dpi from plants inoculated with PVX204 viruses. Images in (A), (B), (C), (G), (H), (I), and (L) show *N. benthamiana* plants inoculated with wild-type and mutant PVX204 viruses. GFP is visualized using a UV lamp. Images in (D), (E), (F), (J) and (K) are upper non-inoculated leaves of *N. tabacum* plants inoculated with wild-type and mutant PVX204 viruses and were taken using an epifluorescence microscope and a 10X objective lens. (A) and (D) PVX204.Δ25 inoculated plants. (B) and (E) PVX204 inoculated plants. (C) and (F), PVX204.TGBp1m4 inoculated plants. (G) and (J) PVX204.TGBp1m3 inoculated plants. (H) and (K) PVX204.TGBp1m5 inoculated plants. (I) Asymptomatic plant inoculated with pPVX204.Δ25. (L) Mosaic symptoms due to systemic PVX204 infection

Table 2. Systemic movement of PVX TGBp1 mutants in *N. tabacum* and *N. benthamiana*[a].

PVX construct	*N. benthamiana*	*N. tabacum*
pPVX204	100%(14/14)	100%(14/14)
pPVX204.Δ25	0 (0/6)	0 (0/6)
pPVX204.TGBp1m1	ND	ND
pPVX204.TGBp1m2	0 (0/8)	0 (0/12)
pPVX204.TGBp1m3	100(8/8)[b]	0 (0/12)
pPVX204.TGBp1m4	100 (8/8)[b]	100 (12/12)
pPVX204.TGBp1m5	0 (0/8)	0 (0/12)
pPVX204.TGBp1m6	0 (0/8)	0 (0/12)
pPVX204.TGBp1m7	0 (0/8)	0 (0/12)
pPVX204.TGBp1m8	0 (0/8)	0 (0/12)
pPVX204.TGBp1m9	ND	ND
pPVX204.TGBp1m11	ND	ND
pPVX204.TGBp1m12	0 (0/8)	0 (0/12)
pPVX204.TGBp1m13	0 (0/8)	0 (0/12)

[a] Percentages of *N. benthamiana* and *N. tabacum* plants in which pPVX204, pPVX204.Δ25, and pPVX204.TGBp1 mutants were observed in systemic tissue. Proportions of plants with systemic infection relative to the total number of plants inoculated are in parentheses.

[b] In *N. benthamiana*, systemic accumulation of PVX204.TGBp1m3 was detected at 21 dpi, PVX204.TGBp1m4 was detected at 10 dpi, and PVX204 was detected between 7 and 10 dpi.

Mutations alter cell-to-cell movement of GFP:TGBp1 in tobacco leaves

In previous studies the pRTL2-GFP:TGBp1 plasmids were biolistically delivered to *N. benthamiana* leaves and *N. tabacum* leaves and it was shown that GFP:TGBp1 proteins moved between adjacent cells in both *Nicotiana* species (Yang et al., 2000). The biolistic delivery technique delivers plasmids to single tobacco leaf epidermal cells. An epifluorescence microscope was used to monitor the spread of GFP fluorescence between adjacent cells. Fluorescence was monitored 24 hours post bombardment (hpb).

In this study, experiments were conducted to determine if the same mutations introduced into the PVX genome affect the ability of TGBp1 to move from cell to cell. Mutations were introduced into pRTL2-GFP:TGBp1 plasmids and these plasmids were biolistically bombarded to *N. benthamiana* and *N. tabacum* leaves (Tables 3 and 4). Cell-to-cell movement of fluorescence was monitored 24 hpb. Representative examples of protein accumulation in single or multiple cells are presented in Fig. 4. As in previous studies conducted in our laboratory (Yang et al., 2000; Krishnamurthy et al., 2002; Krishnamurthy et al., 2003), the proportion of sites that were multiple cell clusters was recorded as evidence of protein cell-to-cell movement (Tables 3 and 4).

As a control, plasmids containing GFP only were also bombarded to *N. benthamiana* and *N. tabacum* leaves. GFP accumulated primarily in single epidermal cells in *N. benthamiana* or *N. tabacum* leaves that were bombarded with pRTL2-GFP plasmids (Fig. 4) (Tables 3 and 4). On rare occasions GFP was detected in two adjacent cells. Similar background levels were reported in related studies, and might occur on occasions when plasmids were delivered to neighboring cells (Itaya et al., 1997; Krishnamurthy et al., 2002; Yang et al., 2000).

As in a previous study, GFP:TGBp1 was detected in multiple cell clusters in *N. benthamiana* and *N. tabacum* source leaves at 24 hpb (Tables 3 and 4) (Yang et al., 2000). Typically, 36 % and 38%, respectively, of sites containing GFP:TGBp1 were multiple cell clusters (Tables 3 and 4). In all leaves, the proportion of cell clusters containing GFP:TGBp1 was significantly greater than the proportion of cell clusters containing GFP ($P<0.05$) (Tables 3 and 4).

A pRTL2-GFP
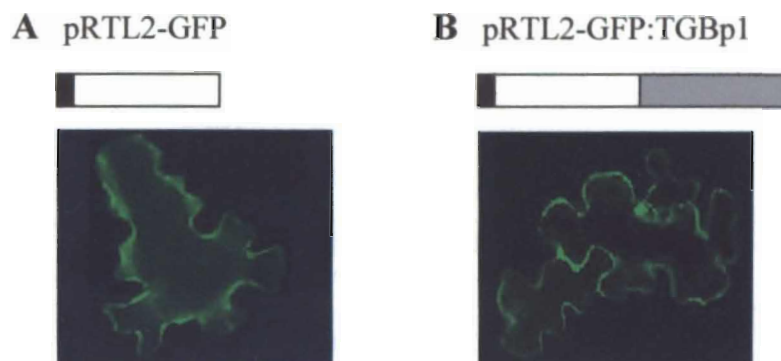
B pRTL2-GFP:TGBp1

Fig. 4. Images of fluorescing cells expressing GFP or GFP:TGBp1. (A) Schematic representation of pRTL2-GFP containing the CaMV 35S promoter (black box) and GFP (white box) used to bombard tobacco epidermal cells. A single epidermal cell expressing GFP is presented. (B) Schematic representation of pRTL2-GFP:TGBp1 and an image of tobacco epidermal cells containing GFP:TGBp1. In this example fluorescence was detected in three adjacent cells.

Mutant pRTL2-GFP:TGBp1 plasmids were biolistically bombarded to *N. benthamiana* and *N. tabacum* leaves. Statistical analyses were conducted to compare the proportions of cell clusters containing GFP, GFP:TGBp1, and each mutant GFP:TGBp1. In *N. benthamiana* leaves, mutants were placed in two classes based on statistical comparisons of their movement activities. In *N. benthamiana* leaves the proportions of sites containing GFP:TGBp1, GFP:TGBp1m1 through GFP:TGBp1m9 were between 30% and 40% (Table 3). The proportions of sites containing wild type or mutant GFP:TGBp1m1 through GFP:TGBp1m9 proteins in multiple cell clusters were not significantly different ($P<0.05$) and were categorized as class I mutants (Table 3).

Mutations m11, m12, and m13 reduced the frequency in which proteins were detected in multiple adjacent cells but did not completely inhibit protein cell-to-cell movement (Table 3). The proportions of multiple cell clusters containing GFP:TGBp1m11 was 23%, GFP:TGBp1m12 was 19%, and GFP:TGBp1m13 was 11% (Table 3) . The proportions of sites that were cell clusters containing GFP:TGBp1m12, and GFP:TGBp1m13 were significantly different from the proportions of sites containing GFP:TGBp1 ($P<0.05$) and were categorized as class II mutants. The proportions of sites that were cell clusters containing GFP:TGBp1m11 were similar to both class I and class II mutants. These data suggest that in *N. benthamiana* leaves mutations m11, m12, and m13 likely affect protein cell-to-cell movement.

In comparing the effects of mutations on protein and virus movement in *N. benthamiana* plants, there does not appear to be a correlation between the effects of mutations on protein and virus movement. All mutations either had no effect or a partial effect on GFP:TGBp1 movement. However, most mutations except m3 and m4 had an

effect on virus movement. These data suggest that the mutations likely disrupt other activities of TGBp1 required for virus cell-to-cell movement.

Table 3. Proportion multiple cell clusters in *N. benthamiana* leaves.[a]

Constructs	Multiple comparison[b]	I	Class[c] II	III
GFP	e			3% (3/102)
GFP:TGBp1	ab	36% (114/321)		
GFP:TGBp1m1	ab	34 (31/90)		
GFP:TGBp1m2	a	37 (30/81)		
GFP:TGBp1m3	a	42 (34/82)		
GFP:TGBp1m4	ab	36 (33/92)		
GFP:TGBp1m5	abc	30 (27/90)		
GFP:TGBp1m6	a	40 (63/159)		
GFP:TGBp1m7	a	41 (47/115)		
GFP:TGBp1m8	ab	33 (30/90)		
GFP:TGBp1m9	ab	32 (28/88)		
GFP:TGBp1m11[d]	bc	23 (21/90)		
GFP:TGBp1m12	cd		19 (18/97)	
GFP:TGBp1m13	d		11 (10/90)	

[a] Percentages of fluorescent cell clusters observed 1 dpb in source leaves are indicated. Multiple cell clusters are defined as two or more adjacent cells showing GFP fluorescence. The total numbers of fluorescent sites that are multiple-cell clusters relative to the total number of fluorescent sites are in parentheses.

[b] Values followed by the same lowercase letter within each column are not significantly different using Fisher's Exact Test at P>0.05.

[c] Values were divided into classes I, II, and III , based on statistical similarities.

[d] GFP:TGBp1m11 was similar to values in both classes I and II.

The effects of the 12 mutations in GFP:TGBp1 on protein cell-to-cell movement in *N. tabacum* leaves was different from that in *N. benthamiana* leaves. Statistically, the mutant proteins could be divided into five classes based on the frequency of movement in *N. tabacum* leaves. The first class contains wild-type GFP:TGBp1 and GFP:TGBp1m4. The proportion of multiple cell clusters containing either GFP:TGBp1 and GFP:TGBp1m4 was 38%, and were not significantly different ($P>0.05$) from one another.

The second class consisted of GFP:TGBp1m3 and GFP:TGBp1m5. The proportions of sites containing GFP:TGBp1m3 or GFP:TGBp1m5 in multiple cell clusters were 17% and 22% respectively. Statistically the values for these two mutants were similar to some values obtained for mutants in class I and class III.

Class III consisted of GFP:TGBp1m1, GFP:TGBp1m2, and GFP:TGBp1m6. The proportions of sites containing each of these mutants were between 11 and 13% and were not significantly different ($P>0.05$). Class IV consisted of GFP, GFP:TGBp1m7, GFP:TGBp1m8, GFP:TGBp1m9, GFP:TGBp1m11, and GFP:TGBpm12; the proportion of multiple cell clusters of these mutations were between 8% and 10% and were not significantly different ($P>0.05$) from one another. Active transport of class III and class IV mutants either occurred at low frequencies or, as in the case of GFP, moved rarely from cell to cell. Class V consisted only of GFP:TGBp1m13. The proportion of sites containing GFP:TGBp1m13 in multiple cell clusters was 3% and this was significantly different from the proportion of sites containing GFP:TGBp1 in multiple cells clusters (38%). Moreover, the proportion of sites containing GFP:TGBp1m13 in multiple cell clusters was significantly less than the proportion of sites containing GFP in multiple cell

clusters (P<0.05). These data suggest that the m13 mutation completely eliminated protein cell-to-cell movement.

Comparing the effects of mutations on GFP:TGBp1 and PVX movement in *N. tabacum* leaves, any mutation that altered the frequency of GFP:TGBp1 cell-to-cell movement also inhibited virus cell-to-cell movement. Only m4 had no effect on protein and virus cell-to-cell movement in *N. tabacum* leaves. All remaining mutants had an effect on protein and virus cell-to-cell movement in *N. tabacum* leaves, regardless of the statistical class used to categorize them. These data suggest that in *N. tabacum*, mutations altering the ability of TGBp1 to move from cell to cell also affect the ability of PVX to move from cell to cell.

Table 4. Proportion of multiple cell clusters in *N. tabacum* leaves.[a]

Construct	Multiple comparison[b]	Class[c] I	II	III	IV	V
GFP	ef					6%(10/158)
GFP:TGBp1	a	38%(43/114				
GFP:TGBp1m1	cd			13%(26/207)		
GFP:TGBp1m2	cd			12 (24/202)		
GFP:TGBp1m3	bc		17 (33/200)			
GFP:TGBp1m4	a	38 (56/148)				
GFP:TGBp1m5	ab		22 (33/150)			
GFP:TGBp1m6	cde			11 (23/201)		
GFP:TGBp1m7	de				8 (17/215)	
GFP:TGBp1m8	de				10 (23/233)	
GFP:TGBp1m9	de				8 (17/201)	
GFP:TGBp1m11	de				10 (28/285)	
GFP:TGBp1m12	cde				10 (20/202)	
GFP:TGBp1m13	f					3 (6/201)

[a]Percentages of fluorescent cell clusters observed 24 hpb in source leaves are indicated. Multiple-cell clusters are defined as two or more adjacent cells showing GFP fluorescence. The total numbers of fluorescent sites that are multiple-cell clusters relative to the total number of fluorescent sites are in parentheses.

[b]Values followed by the same lowercase letter within each column are not significantly different using Fisher's Exact Test at $P < 0.05$.

[c]Values were divided into classes I, II, III , IV, and V based on statistical similarities.

[d]GFP:TGBp1 mutants which had values that were statistically similar to values in

Two mutant GFP:TGBp1 proteins move cell to cell in TGB600 leaves

Line TGB600 transgenic tobacco expresses TGBp2 and TGBp3 proteins and can complement cell-to-cell movement of PVX viruses containing mutations in TGBp2 and/or TGBp3 (Verchot et al., 1998). It was also shown previously that while GFP:TGBp1 moves from cell-to-cell in non-transgenic *N. tabacum*, movement of GFP:TGBp1 is restricted in TGB600 tobacco (Krishnamurthy et al., 2002). From this work it was suggested that either the triple gene block proteins interact directly to form a complex that restricts cell-to-cell movement of GFP:TGBp1, or TGBp2 and TGBp3 reside in PD blocking transport of GFP:TGBp1 indirectly (Fig. 5) (Krishnamurthy et al., 2002).
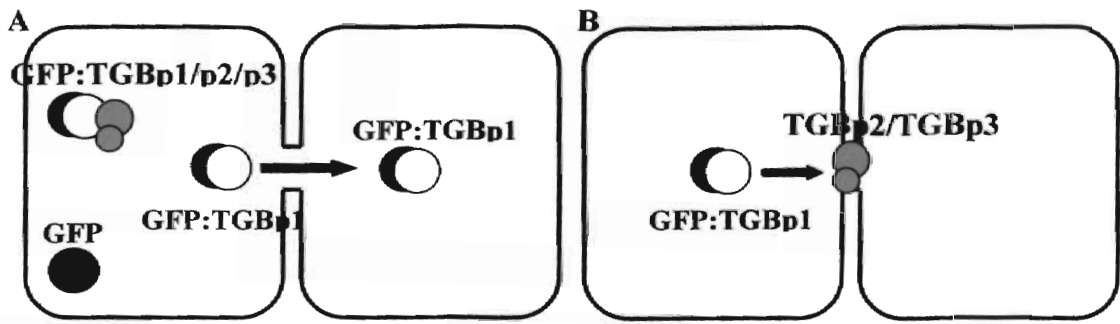
Fig. 5. Model detailing possible interactions among the triple gene block proteins that might regulate cell-to-cell movement of TGBp1. The two white boxes are plant cells connected by PD. (A) TGBp1 (white circle) fused to GFP (black crescent) moves (indicated with black arrow) on non-transgenic tobacco. GFP (black circle) does not move from cell to cell. GFP:TGBp1 does not move cell-to-cell in TGB600 leaves expressing TGBp2 and TGBp3. TGBp2/TGBp3 (gray circles) might bind GFP:TGBp1 preventing its cell-to-cell movement. (B) Alternatively, TGBp2/TGBp3 might reside in PD in TGB600 leaves and thereby blocks cell-to-cell movement of GFP:TGBp1 (Krishnamurthy et al., 2002).

Plasmids expressing mutant GFP:TGBp1 proteins were bombarded to TGB600 tobacco plants. If cell-to-cell movement of GFP:TGBp1 is blocked because it forms a complex with TGBp2 and TGBp3, then mutations disrupting these interactions should promote cell-to-cell movement of GFP:TGBp1. If on the other hand, TGBp2 and TGBp3 reside in PD and thereby block movement of GFP:TGBp1, then no mutation should have an effect on GFP:TGBp1 movement. Wild-type and mutant pRTL2-GFP:TGBp1 plasmids were biolistically bombarded to TGB600 leaves. As a controls pRTL2-GFP plasmids were also bombarded to TGB600 leaves. Statistical analyses were conducted to compare the proportions of cell clusters containing GFP, GFP:TGBp1, and each mutant GFP:TGBp1.

GFP and GFP:TGBp1 accumulated primarily in single cells. On rare occasions GFP was detected in two adjacent cells. Similar to wild-type GFP:TGBp1, most mutations (TGBp1m2, m3, m4 through m13) accumulated in single cells. Only mutants GFP:TGBp1m1 and GFP:TGBp1m5 were detected in multiple cell clusters. The proportions of sites containing GFP:TGBp1m1 or GFP:TGBp1m5 in multiple cell clusters was 15% and 22%, respectively. Statistically the values for these two mutants were significantly different from values obtained for GFP or GFP:TGBp1 (P<0.05). These data suggest that the m1 and m5 mutations disrupted TGBp1 interactions with TGBp2 and TGBp3 thereby promoting cell-to-cell movement of TGBp1.

The m1 and m5 mutations inhibited movement of PVX204 in *N. tabacum* and *N. benthamiana* plants. While these mutations promoted protein cell-to-cell movement in TGB600 leaves these data suggest that interactions between TGBp1, TGBp2, and TGBp3 might be important for virus cell-to-cell movement.

Table 5. Proportion of multiple cell clusters in *N. tabacum* TGB600 leaves.[a]

Constructs	Multiple comparison[b]	Class[c] I	II	III
GFP	cd			6%(10/165)
GFP:TGBp1[d]	bc			8 (13/161)
GFP:TGBp1m1	ab		15%(31/207)	
GFP:TGBp1m2[d]	bc			9 (19/201)
GFP:TGBp1m3	cd			6 (6/100)
GFP:TGBp1m4	cd			6 (9/141)
GFP:TGBp1m5	a	22%(26/117)		
GFP:TGBp1m6[d]	bc			8 (15/190)
GFP:TGBp1m7	cd			5 (6/123)
GFP:TGBp1m8[d]	bc			8 (8/99)
GFP:TGBp1m9	cd			2 (2/118)
GFP:TGBp1m11[d]	bc			8 (16/200)
GFP:TGBp1m12	cd			7 (8/122)
GFP:TGBp1m13	d			3 (4/145)

[a] Percentages of fluorescent cell clusters observed 24 hpb in source leaves are indicated. Multiple-cell clusters are defined as two or more adjacent cells showing GFP fluorescence. The total numbers of fluorescent sites that are multiple-cell clusters relative to the total number of fluorescent sites are in parentheses.

[b] Values followed by the same lowercase letter within each column are not significantly different using Fisher's Exact Test at $P < 0.05$.

[c] Values were divided into classes I, II, and III based on statistical similarities.

[d] GFP:TGBp1 mutants which had values that were statistically similar to values in adjacent classes.

Wild-type and mutant GFP:TGBp1 do not move from cell to cell in CP-transgenic tobacco

Line CP transgenic tobacco complements cell-to-cell movement of PVX viruses containing mutations in the PVX CP (Spillaine et al., 1997). It was also shown previously that while GFP:TGBp1 moves from cell to cell in non-transgenic *N. tabacum*, movement of GFP:TGBp1 is restricted in CP transgenic tobacco leaves (Krishnamurthy et al., 2002). As in studies using TGB600 transgenic tobacco, it was suggested that TGBp1 and CP interact directly to form a complex which restricts cell-to-cell movement of GFP:TGBp1, or CP reside in PD blocking transport of GFP:TGBp1 (Krishnamurthy et al., 2002).

Plasmids expressing GFP, wild-type GFP:TGBp1, or mutant GFP:TGBp1 proteins were bombarded to CP tobacco leaves. Statistical analyses were conducted to compare the proportions of cell clusters containing GFP, GFP:TGBp1, and each mutant GFP:TGBp1. As expected, GFP and GFP:TGBp1 each accumulated primarily in single cells in CP tobacco leaves (Krishnamurthy et al., 2002). Furthermore, each of the mutant GFP:TGBp1 proteins accumulated primarily in single cells (Table 6). The proportions of sites containing GFP:TGBp1m2 or GFP:TGBp1m7 in multiple cell clusters was 12% suggesting there may be some protein movement in leaves bombarded with these plasmids. The proportions of sites containing GFP:TGBp1m6 and GFP:TGBp1m11 were both 0%. All values were generally low. While there may be some statistical differences between mutants it does not appear that any of the mutations restored protein movement to levels seen in non-transgenic plants inoculated with wild-type GFP:TGBp1 (compare Tables 2 and 6). Using mutational analysis, there is no evidence to discern whether CP restricts protein movement by directly binding GFP:TGBp1 or if it acts indirectly at the PD to block movement of GFP:TGBp1.

Table 6. Proportion of multiple cell clusters in CP-transgenic leaves.[a]

Construct	Multiple comparison[b]	
GFP	abc	7%(18/252)
GFP:TGBp1	abc	5 (6/111)
GFP:TGBp1m1	abc	7 (9/130)
GFP:TGBp1m2	a	12 (12/98)
GFP:TGBp1m3	bc	5 (11/236)
GFP:TGBp1m4	bc	4 (5/133)
GFP:TGBp1m5	ab	9 (15/166)
GFP:TGBp1m6	a	10 (18/177)
GFP:TGBp1m7	a	12(23/188)
GFP:TGBp1m8	cd	3 (4/147)
GFP:TGBp1m9	abc	7 (8/117)
GFP:TGBp1m11	ab	10 (15/150)
GFP:TGBp1m12	abc	7 (10/143)
GFP:TGBp1m13	d	0 (0/108)

[a] Percentages of fluorescent cell clusters observed 1 dpb in source leaves are indicated. Multiple cell clusters are defined as two or more adjacent cells showing GFP fluorescence. The total numbers of fluorescent sites that are multiple-cell clusters relative to the total number of fluorescent sites are in parentheses.

[b] Values followed by the same lowercase letter within each column are not significantly different using Fisher's Exact Test at $P < 0.05$.

Mutant GFP:TGBp1 proteins move in TGB100 transgenic tobacco

Experiments were conducted to determine if cell-to-cell movement of mutant GFP:TGBp1 could be altered in TGB100 transgenic tobacco which express the TGBp1 protein. Plasmids expressing GFP, wild-type GFP:TGBp1 or mutant GFP:TGBp1 proteins were biolistically delivered to TGB100 transgenic tobacco leaves (Table 7). In comparing Tables 4 and 7 the proportion of multiple cell clusters containing GFP:TGBp1 in non-transgenic leaves was 38% but in TGB100 leaves the proportion was 17%. These data suggest that the transgenically expressed protein reduced cell-to-cell movement of GFP:TGBp1. Cell-to-cell movement of GFP:TGBp1 might require interactions with cellular factors for PD transport which may have been blocked by the transgenically expressed TGBp1.

Cell-to-cell movement of GFP:TGBp1m1, GFP:TGBp1m7 , GFP:TGBp1m8, GFP:TGBp1m9, GFP:TGBp1m11 was enhanced in TGB100 leaves. In the case of GFP:TGBp1m1, 13% of sites were multiple cell clusters in non-transgenic leaves (Table 4) while 27% of sites were multiple cell clusters in TGB100 leaves (Table 7). While GFP:TGBp1m7, GFP:TGBp1m8, GFP:TGBp1m9, GFP:TGBp1m11 accumulated primarily in single cells in non-transgenic leaves (Table 4), between 17 and 27% of sites were multiple cell clusters in TGB100 leaves expressing these mutant proteins (Table 7). These data indicate that the transgenically expressed TGBp1 protein complemented cell-to-cell movement of these mutant GFP:TGBp1 proteins.

Table 7. Proportion of multiple cell clusters in TGB100 leaves.[a]

Construct	Multiple comparison[b]	Class[c] I	II	III
GFP	c			5%(8/160)
GFP:TGBp1	b		17%(21/124)	
GFP:TGBp1m1	a	27%(40/147)		
GFP:TGBp1m2	c			5 (8/155)
GFP:TGBp1m3[d]	bc			9 (13/141)
GFP:TGBp1m4	-	nd		
GFP:TGBp1m5	-	nd		
GFP:TGBp1m6[d]	bc			10 (14/128)
GFP:TGBp1m7[d]	ab		20 (40/193)	
GFP:TGBp1m8[d]	ab		21 (29/135)	
GFP:TGBp1m9	b		17 (31/184)	
GFP:TGBp1m11[d]	ab		18 (34/179)	
GFP:TGBp1m12[d]	bc			9 (28/147)
GFP:TGBp1m13	c			7 (14/176)

[a]Percentages of fluorescent cell clusters observed 1 dpb in source leaves are indicated. Multiple cell clusters are defined as two or more adjacent cells showing GFP fluorescence. The total numbers of fluorescent sites that are multiple cell clusters relative to the total number of fluorescent sites are in parentheses.

[b]Values followed by the same lowercase letter within each column are not significantly different using Fisher's Exact Test at $P < 0.05$.

[c]Values were divided into classes I, II, and III based on statistical similarities.

[d] Many GFP:TGBp1 mutants had values that were statistically similar to values in adjacent classes.

DISCUSSION

To determine if TGBp1 cell-to-cell movement is essential for virus cell-to-cell movement, the effects of mutations in TGBp1 on protein and virus cell-to-cell movement were compared. Only mutation m4 had no effect on protein or virus cell-to-cell movement in *N. benthamiana* or *N. tabacum*. All other mutations had some effect on protein and/or virus movement

Data obtained in *N. benthamiana* and *N. tabacum* leaves were conflicting. A correlation between the effects of mutations on protein and virus movement did not exist in *N. benthamiana*, while in *N. tabacum* there was a correlation between the effects of mutations on both protein and virus movement. In *N. benthamiana*, mutations m2, m3, m5 through m8 did not significantly affect protein movement but either delayed or eliminated virus movement (compare Tables 2 and 3). In *N. tabacum*, mutations m2, m3, m5 through m8 reduced or eliminated protein cell-to-cell movement and eliminated virus movement (compare Tables 2 and 4). These data suggest that TGBp1 cell-to-cell movement might contribute to, but not be essential for, virus cell-to-cell movement in all host species. Other features of TGBp1 such as PD gating, ATPase activity, or helicase activity might play a greater role in virus movement.

Mutations m12 and m13 are substitution mutations replacing the ATPase active site G-K-S and D-E-Y motifs. Mutations m12 and m13 inhibited virus cell-to-cell movement in both *Nicotiana* species suggesting that ATPase activity is essential for virus cell-to-cell movement. However, the role of the ATPase active sites in protein cell-to-cell movement in both *Nicotiana* species might differ. Mutation m12 and m13 reduce protein cell-to-cell movement in *N. benthamiana* while in *N. tabacum* these mutations eliminated protein movement (compare Tables 3 and 4). ATPase activity might

66

contribute to protein movement in *N. benthamiana* but not in *N. tabacum*. Further experiments are needed to characterize the effects of these mutations on ATP hydrolysis and on protein cell-to-cell movement.

Mutation m5 was interesting because it eliminated interactions with TGBp2 and TGBp3 in TGB600 transgenic *N. tabacum* leaves, eliminated virus movement in both *Nicotiana* species, and had either no effect or a only minor effects on protein cell-to-cell movement in *N. benthamiana* and *N. tabacum* leaves. The effects of the m5 mutation on the ability of TGBp1 to interact with TGBp2 and TGBp3 were profound and suggest that a complex containing TGBp1, TGBp2, and TGBp3 might be essential for virus cell-to-cell movement. While, wild-type GFP:TGBp1 does not move from cell to cell in TGB600 transgenic leaves expressing TGBp2 and TGBp3, GFP:TGBp1m5 did move from cell to cell in TGB600 leaves. Data presented in Table 6 suggest that the m5 mutation restored TGBp1 cell-to-cell movement by disrupting the ability of TGBp1 to interact with TGBp2 and TGBp3. Since this m5 mutation also eliminated virus cell-to-cell movement in both *Nicotiana* species (Table 2), these data suggest that a complex containing TGBp1, TGBp2, and TGBp3 is essential for virus cell-to-cell movement.

Evidence obtained with the m5 mutation also suggests that TGBp1 cell-to-cell movement might contribute to, but not be essential for, virus cell-to-cell movement. First, evidence that a complex containing TGBp1, TGBp2, and TGBp3 does not move from cell-to-cell in *N. tabacum* (Krishnamurthy et al., 2002) suggests that movement of TGBp1 through the PD might not be essential for virus movement. TGBp2 and TGBp3 are ER-associated proteins and might bind TGBp1 preventing it from movement through PD (Krishnamurthy et al. 2003; Mitra et al., 20003). Second, in *N. benthamiana* the proportions of sites containing GFP:TGBp1 and GFP:TGBp1m5 were similar, while in

67

N. tabacum the proportions of sites containing GFP:TGBp1m5 in multiple cell clusters was 22% and the proportions of sites containing GFP:TGBp1 in cell clusters was 38%. While cell-to-cell movement of GFP:TGBp1m5 was only slightly impaired in *N. tabacum*, the mutation eliminated virus cell-to-cell movement further supporting the notion that cell-to-cell movement of TGBp1 might not be essential for virus cell-to-cell movement.

The effects of mutations on interactions between TGBp1 and CP were studied. None of the mutations in TGBp1 restored protein cell-to-cell movement in CP transgenic tobacco. It was shown in a previous study that CP accumulates inside PD (Santa Cruz 1998) but does not induce PD gating. One possibility is that CP acts as a factor inside PD to down regulate PD gating activities of TGBp1 without directly binding TGBp1.

Plasmids expressing wild-type and mutant GFP:TGBp1 proteins were also bombarded to TGB100 transgenic tobacco plants expressing the wild-type TGBp1 protein. Cell-to-cell movement of mutants m7 through m9 and m11 was enhanced in TGB100 leaves in comparison to non-transgenic leaves. These mutations lie in the C-terminal half of TGBp1. Mutants m2, m3, m6, m12, and m13 reside in the N-terminal ATPase domain and cell-to-cell movement was not restored by the wild-type protein. One possibility is that ATPase activity is required in cis for protein cell-to-cell movement. Alternatively, TGBp1 could form dimers that move from cell to cell and mutations in the N-terminal half of the protein disrupt dimerization. In a yeast two hybrid assay we have obtained evidence that TGBp1 dimerizes (unpublished data). It is also known that TGBp1 forms crystalline inclusion bodies in plant cells (Davies et al., 1993) and so TGBp1 dimerization or oligomerization might not be an essential feature.

Further direct experimentation is needed to determine if these mutations affect dimerization or oligomerization of TGBp1.

A model was proposed suggesting (Lough et al., 1998) that TGBp1 binds viral RNA and forms a complex with the triple gene block proteins to carry virus through PD. Evidence presented in a related study indicates TGBp1 is not an RNA binding protein but an RNA helicase. Moreover TGBp1, TGBp2, and TGBp3 form complexes, which do not move from cell-to-cell, and mutations in TGBp1, which have no or minimal effects on TGBp1 cell-to-cell movement but affect virus movement. Taken together, RNA binding and movement through the PD might not be the primary functions of TGBp1 toward virus cell-to-cell movement.

It has been recently shown that TGBp1 suppresses gene silencing (Voinnet et al., 2000). We do not know which activities of TGBp1 play a role in suppression of gene silencing versus promoting virus cell-to-cell movement. It is possible that protein cell-to-cell movement is more important for suppression of gene silencing than for virus cell-to-cell movement. In respect to the gene silencing pathway, it has been shown that TGBp1 blocks systemic spread of the silencing signal (Voinnet et al., 2000). Early in virus infection, TGBp1 might move from cell-to-cell ahead of virus infection to suppress gene silencing and thereby promote virus infection.

CHAPTER IV

TGBp1 IS A TEMPERATURE SENSITIVE SUPPRESSOR OF VIRUS INDUCED GENE SILENCING (VIGS)

ABSTRACT

Virus induced gene silencing (VIGS) is an RNA dependent RNA polymerase (RdRP) independent form of post-transcriptional gene silencing (PTGS), a mechanism by which double stranded viral RNA is degraded in a highly sequence specific manner. Viruses have evolved to counteract this pathway by encoding a suppressor protein. These proteins block VIGS at different points in the pathway. PVX TGBp1 blocks the movement of the systemic silencing signal from the inoculated leaf. Amino acid sequences essential for TGBp1 suppressor function have not been characterized, and it is not known if suppressor function contributes to virus cell-to-cell movement. Mutational analysis was therefore used to characterize sequences that contribute to suppressor function. Wild-type TGBp1 as well as six mutants that contribute to TGBp1 protein and virus cell-to-cell movement were delivered to GFP-transgenic *N. benthamiana* using the *Tomato bushy stunt virus* (TBSV) and defective interfering RNA plus GFP (DI-P) vector. This delivery mechanism mimics a natural virus infection by co-delivering the viral suppressor with inducer RNA. The wild-type TGBp1 suppressor, which suppressed VIGS at 25°C, lost suppressor function at 28°C. Thus, TGBp1 suppressor function is temperature sensitive. TGBp1m3 disrupted suppressor function at both temperatures suggesting that the sequences disrupted by this insertion contribute to the suppression of VIGS by TGBp1. Data obtained from the other mutations tested was inconclusive.

INTRODUCTION

In plants, post-transcriptional gene silencing (PTGS) is a mechanism that inactivates gene expression in the cytoplasm by blocking mRNA accumulation (Plasterk, 2002). PTGS has been studied using green fluorescent protein (GFP)-transgenic plants (Angell and Baulcombe, 1997). Expression of the GFP transgene was silenced in *N. benthamiana* plants infected with *Agrobacterium tumefaciens* modified to contain the same GFP gene. In this case, the *Agrobacterium tumefaciens* GFP gene triggered the PTGS mechanism causing the degradation of homologous GFP-RNAs (Angell and Baulcombe, 1997). Transgene derived, GFP-encoding RNAs are targeted for degradation, thereby silencing GFP expression throughout the plant.

Viruses have been described as both initiators and targets of PTGS (Kumagi et al., 1995; Lindbo et al., 1993; Ruiz et al., 1998). PTGS triggered by virus infection is known as virus induced gene silencing (VIGS). When viral vectors expressing the GFP gene were inoculated to GFP transgenic *N. benthamiana*, virus and transgene accumulation were silenced. The process of RNA silencing in VIGS is similar to that of PTGS; homology between viral and transgene derived RNAs trigger the VIGS or PTGS mechanism which degrades RNAs in a sequence specific manner (Baulcombe 1996; Lindbo et al., 1993). The PTGS mechanism has been triggered in some non-transgenic plants inoculated with plant viruses suggesting that this PTGS may function as a natural anti-virus defense mechanism (Plasterk, 2002).

The PTGS mechanism produces a systemic signal that blocks systemic virus accumulation throughout the plant (Voinnet et al., 2000). In response to this plant defense mechanism, plant viruses have evolved the capacity to block the PTGS mechanism. The potyvirus HC-Pro protein and the *Cucumber mosaic virus* 2b proteins were among the

71

first identified as suppressors of PTGS in *N. benthamiana* (Anandalakshmi et al., 1998; Brigneti et al., 1998). More recently the PVX TGBp1 protein was shown to be a suppressor of PTGS by blocking spread of the systemic signal of gene silencing from the initially inoculated leaves (Voinnet et al., 2000).

In this study, mutations were introduced into the TGBp1 protein to identify sequences within TGBp1 contributing to silencing suppression. A novel co-delivery system consisting of *Tomato bushy stunt virus* (TBSV) vector and a modified defective interfering RNA (DI-P) that can induce silencing of GFP transgenes (Hou and Qiu, 2003) was employed to study the effects of the mutations on TGBp1 suppressor activity. TBSV viruses encoding mutant TGBp1 proteins were inoculated to GFP-transgenic *N. benthamiana* plants. Results of this study suggest that the silencing suppression activity of TGBp1 might be temperature sensitive.

MATERIALS AND METHODS

Plasmids and bacterial strains

The plasmid pHST2-14 was obtained from Dr. Wenping Qiu (Southwest Missouri State University, MO) and contains a cDNA copy of the TBSV genome with an inactivated P19 gene silencing suppressor protein (Hou and Qui, 2003, Scholthof et al, 1996; Scholthof, 1999) (Fig. 1B). The TBSV genome is located in pHST2-14 adjacent to the bacteriophage T7 promoter (Scholthof et al., 1996). The plasmid pHST2-14 was used in this study as a vector to express the PVX TGBp1 gene.

The TBSV DI-P was used as a silencing inducer of the homologous GFP transgene in *N. benthamiana* plants (Hou and Qui, 2003). DI-P contains the 3' portion of the GFP gene (Hou and Qui, 2003) (Fig 1B).

TBSV.GFP was prepared previously (Qui et al., 2002) and contains the GFP gene inserted into the TBSV genome replacing the viral CP gene (Fig. 1B). Wild-type and the six mutant PVX TGBp1 genes were introduced into the TBSV genome to test their function as a suppressor of PTGS (Fig 1B). The six of the previously described TGBp1 mutations (m1, m3, m5, m8, m9, m12) introduced into TBSV were either insertion or substitution mutations. Wild-type and mutant TGBp1 genes were cloned into the pHST2-14 vector using *Xho*I and *Sac*I restriction sites, which are located at each end of the TBSV coat protein gene (Fig. 1B). Wild-type and mutant TGBp1 genes were PCR amplified from pRTL2-GFP:TGBp1 constructs using the a forward primer (GCG GTC GAC CGA TGG ATA TTC TCA TCA GTA GT) that contains an *Xho*I site (underlined) and a reverse primer (GCG GAG CTC CGT CAG CCT ATG GCC CTG CGC GG) that contains a *Sac*I site (underlined). PCR products and pHST2-14 plasmids were digested with *Xho*I and *Sac*I and then were ligated together. All plasmids were constructed and used to transform *Escherichia coli* strain JM109 using methods described in Sanbrook et al. (1989).

In vitro transcription and plant inoculations

Infectious transcripts of TBSV containing GFP, wild-type TGBp1, mutant TGBp1, and DI-P were prepared and used to inoculate non-transgenic *N. benthamiana* or GFP-expressing transgenic *N. benthamiana* plants. Plasmids were lineared with *Sma*I and *in vitro* transcription reactions were carried out by mixing: 0.25 µg of linearized DNA, 5 µl of 5X T7 transcription buffer, 1.0 µl 0.1 M DTT, 0.5 µl SUPERase·In™ ribonuclease inhibitor (20U/µl)(Ambion, Austin, TX), 2.5 µl of an NTP mixture containing 5mM ATP, CTP, UTP, and GTP (Pharmacia-Pfizer, Mississauga, Ontario,

Canada), 0.7 µl of T7 polymerase, and nuclease-free water to a final volume of 25 µl. The reactions were incubated for one and a half hour at 37^0C (Qiu et al., 2002).

TBSV.GFP, and TBSV plus TGBp1 transcripts were mixed 2:1 with DI-P transcripts (Qiu et al. 2002). The mixed transcripts were combined 1:1 with inoculation buffer (50 mM K_2HPO_4 (pH 9.0), 50 mM glycine, 1.0% bentonite, and 1.0% celite). An inoculation buffer (50 mM K_2HPO_4 (pH 9.0), 50 mM glycine, 1.0% bentonite, and 1.0% celite) was added 1:1 to the combined TBSV and DI-P mix. The buffer/transcript mix was then rub-inoculated to non-transgenic and GFP-transgenic *N. benthamiana* leaves. Two leaves per plant, and three plants, were inoculated in each experiment. Experiments were repeated three times for a total of 9 plants per construct.

The transgenic *N. benthamiana* line 16C, used in this study, was provided by Dr. Wenping Qiu (Department of Fruit Science, Southwest Missouri State University, Mountain Grove, MO, USA) and is homozygous for the GFP transgene at a single locus (Ruiz et al., 1998; Voinnet et al., 1999). Line 16C is widely used for studying PTGS (Hou and Qiu, 2003; Ruiz et al., 1998; Qiu et al., 2002; Voinnet et al., 1999; Voinnet et al., 2000). Non-transgenic and line 16C plants were grown in a growth chamber at 24°C with 14 h light and 10 h dark.

Visualization of GFP

The plants were initially monitored using a hand-held model B-100 BLAK-RAY long wave ultraviolet lamp (Ultraviolet Products, Upland, CA) every 3 days for up to 21 days post inoculation (dpi). Later, plants were monitored and scored for systemic silencing between 15 and 21 dpi and GFP expression was recorded with a Sony Digital Still Camera model DSC-F717 (Sony Corporation of America, New York City, New York).

GFP expression was also monitored in detached leaves of inoculated plants using a Nikon E600 epifluorescence microscope (Nikon Inc., Dallas, TX) with a Nikon B2A filter cube (containing a 470-490 nm excitation filter, a DM505 dichroic mirror, and a BA520 barrier filter). Images were captured using the Optronics Magnafire camera (Intelligent Imaging Innovations, Inc., Denver, CO) attached to the Nikon E600 microscope. Images were compiled using Adobe Photoshop 4.0 software (Adobe Systems Inc. San Jose, CA).

A. TBSV genome

B. TBSV constructs

pHST2-14

XhoI/SalI   GFP   SacI

TGBp1

TGBp1m

DI-P
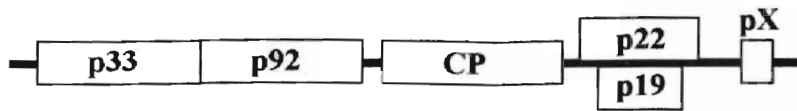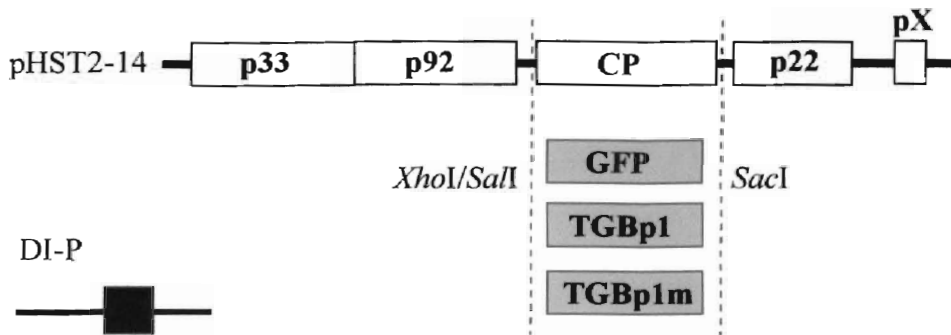
Fig. 1. Diagrammatic representation of the TBSV genome, TBSV, and DI-P constructs. The lines indicate noncoding regions and boxes indicate protein open reading frames. **A.** Schematic representation of the TBSV genome. The P33 and P92 proteins comprise the viral replicase. The P22 is the viral MP, P19 is a gene silencing suppressor, and pX has no known function. **B.** Schematic representation of pHST2-14 derived plasmids and DI-P. pHST2-14 has the P19 silencing suppressor protein deleted from the TBSV genome (Qui et al., 2002; Hou and Qui., 2003). *Xho*I and *Sac*I restriction sites flank the CP open reading frame. In TBSV.GFP, TBSV.TGBp1, or TBSV.TGBp1m (1, 3, 5, 8, 9, and 12), the GFP, TGBp1 or mutant TGBp1 open reading frames (gray boxes) were inserted into the TBSV genome replacing the CP open reading frame. DI-P is a defective interfering (DI) RNA associated with TBSV, which was modified to contain the 3' portion of GFP (black box) (Qui et al., 2002).

RESULTS

TBSV plus DI-P induces gene silencing in *N. benthamiana*

The pHST2-14 plasmid encodes the TBSV viral genome (Fig. 1B), which has been modified to express foreign genes. The viral CP is dispensable for virus replication and cell-to-cell movement in *N. benthamiana* (Scholthof et al., 1993) and therefore can be replaced by foreign genes (Fig. 1C). The p19 protein of TBSV is a suppressor of gene silencing (Qui et al., 2002). To study the effects of foreign genes on PTGS, the p19 gene was deleted from the viral genome in pHST2-14. DI-P is a DI-RNA of TBSV that was modified to contain a portion of the GFP gene that can induce gene silencing in GFP-expressing transgenic *N. benthamiana* inoculated with TBSV plus DI-P (Fig. 1C) (Qui et al., 2002). GFP silencing can be monitored using a hand held UV lamp. In regions were virus is accumulating, GFP expression is silenced and only red fluorescence due to chlorophyll can be observed (Hou and Qui, 2003). In previous reports, red spots were first observed in inoculated leaves by 6 dpi (Hou and Qui, 2003). Red spots represent virus infection foci where virus infection and gene silencing initiate. These spots expanded to surrounding tissue over a period of 9 days. As virus spreads systemically red fluorescence is observed along the veins in upper leaves. Red fluorescence, indicating systemic silencing of GFP, was prevalent in upper leaves by 10 dpi (Qui et al., 2002; Hou and Qui, 2003).

To determine if a mutant TBSV lacking the p19 gene has an effect on PTGS, TBSV.GFP plus DI-P transcripts were inoculated to GFP-transgenic *N. benthamiana* plants. TBSV.GFP plus DI-P lacks a suppressor gene and induces silencing; red spots were observed in inoculated leaves by 6 dpi and in upper non-inoculated leaves by 15 dpi (Fig. 2C) (Hou and Qui, 2003). Thus, TBSV.GFP plus DI-P induced gene silencing in

GFP-expressing *N. benthamiana* plants. These data indicate that a mutant TBSV lacking the p19 gene is not itself capable of suppressing PTGS (Fig. 2C) (Hou and Qui, 2003).

TGBp1 silencing suppression activity is temperature sensitive

The PVX TGBp1 gene was inserted into the TBSV genome (Fig. 1B) to determine if the TGBp1 protein can suppress PTGS in GFP-expressing *N. benthamiana* plants. TBSV.TGBp1 and DI-P transcripts were inoculated to GFP-expressing *N. benthamiana* plants and then plants were maintained at 25°C or 28°C. Red fluorescent spots were observed in inoculated leaves between 6 and 9 dpi, indicating that TGBp1 had no effect on PTGS in inoculated leaves (Fig. 2D). Plants were maintained for 21 dpi and GFP expression was unaltered in upper non-inoculated leaves (Fig. 2E). These data confirm a previous study indicating that TGBp1 suppresses PTGS in systemic tissues (Voinnet et al., 2000).

The proportions of plants that displayed gene silencing in the inoculated leaves were similar whether plants were maintained at 25°C or 28°C. However, the proportions of plants displaying systemic gene silencing differed at these two temperatures (Table 1). At 25°C, TGBp1 suppression of systemic silencing in 100% of the inoculated plants but at 28°C suppression was observed in only 50% of the plants analyzed (Table 1; compare Fig 2E and 2F). TBSV.TGBp1 produced similar symptoms on plants inoculated at either temperature. These data suggest that suppressor activity of TGBp1 is temperature sensitive.

Previously in this study mutations were introduced into the TGBp1 coding region of PVX to identify protein sequences important for virus cell-to-cell movement. A select group of mutant genes (TGBp1m1, TGBp1m3, TGBp1m5, TGBp1m8, TGBp1m9, and TGBp1m12) were introduced into the TBSV genome and the effects of these mutations

78

on PTGS were analyzed. Plants were inoculated with mutant TBSV.TGBp1 plus DI-P transcripts and were maintained at 25°C or 28°C.

Mutations m8 and m12 altered TGBp1 suppressor activity at 28°C but not at 25°C. Similar to TBSV.TGBp1, TBSV.TGBp1m8 and TBSV.TGBp1m12 each suppressed PTGS in 100% of plants inoculated at 25°C (Table 1; Fig. 2G). However, at 28°C TBSV.TGBp1m8 and TBSV.TGBp1m12 did not suppress PTGS suggesting that these mutations only affect activities of TGBp1 at the higher temperature.

The TGBp1 mutation, m3, disrupted suppressor activity at both temperatures but did not completely eliminate TGBp1 suppressor activity. TBSV.TGBp1m3 suppressed PTGS at 25°C in 50%, and at 28°C in 33% of plants inoculated (Table 1). Thus a greater proportion of plants showed systemic silencing following inoculation with TBSV.TGBp1m3 than inoculation with TBSV.TGBp1 (Table 1).

TBSV.TGBp1m1, TBSV.TGBp1m5, and TBSV.TGBp1m9 eliminated TGBp1 suppressor activity in plants inoculated at 28°C. Suppression was evidenced by green fluorescence in upper non-inoculated leaves (Table 1).
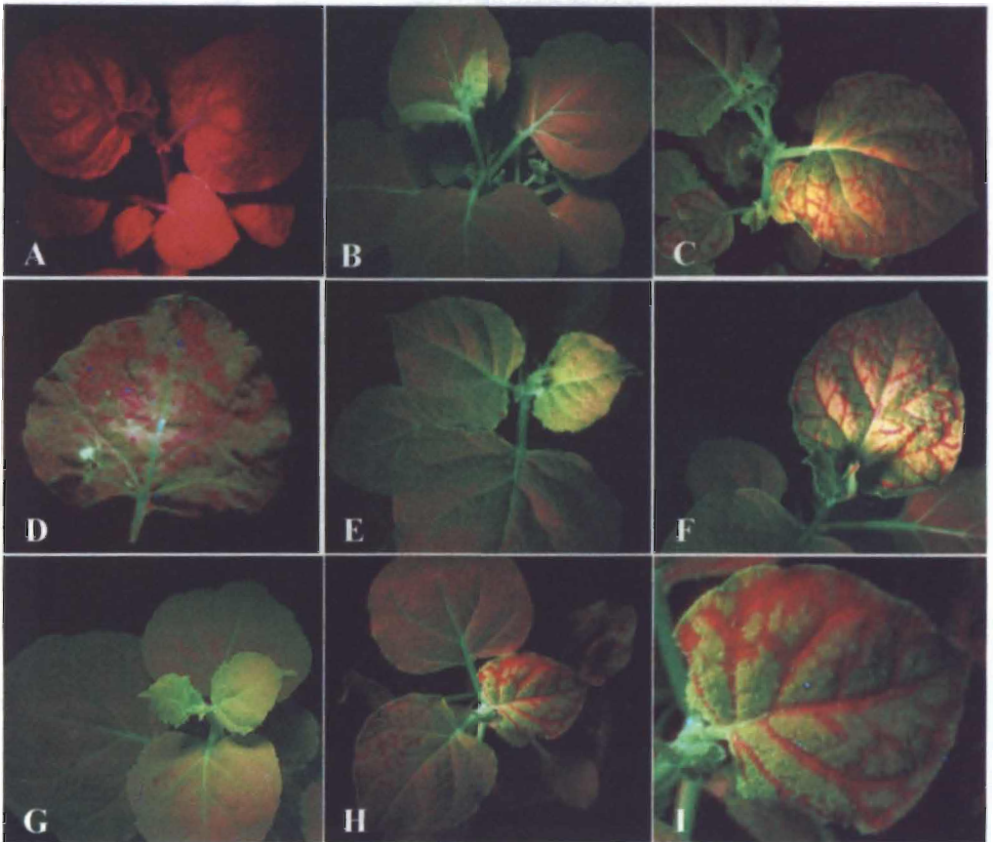
Fig. 2. Images of plants inoculated with TBSV.GFP plus DI-P or with TBSV expressing wild-type or mutant TGBp1 plus DI-P. Green fluorescence due to GFP expression was observed using a hand held UV lamp. Red fluorescence was due to chlorophyll in the leaves. (A) Non-transgenic *N. benthamiana* plant. (B) Green fluorescing GFP transgenic *N. benthamiana* plant. (C) TBSV.GFP induced systemic PTGS. GFP is silenced along the veins, which appear red. (D, E, F) TBSV.TGBp1 plus DI-P inoculated GFP transgenic *N. benthamiana* plants. (D) Red fluorescent spots indicating GFP silencing in inoculated leaves. (E) GFP fluorescence in upper non inoculated leaves of plants at 25°C, however in some plants held at 28°C (F) PTGS in upper leaves was not suppressed by TBSV.TGBp1. (G, H, I) Example of a GFP transgenic *N. benthamiana* inoculated with TBSV.TGBp1m8, which suppressed systemic PTGS at 25°C (G) but not at 28°C (H). (I) Image of a silenced upper, non-inoculated leaf in a plant inoculated with TBSV.TGBp1m8 plus DI-P showing red fluorescence along the veins.

Table 1. Suppression of VIGS in GFP-transgenic *N. benthamiana* plants by TGBp1 and mutant TGBp1 at 25°C and 28°C.[a]

Constructs	25°C	28°C
TBSV.GFP + DI-P	nd	0%(0/3)
TBSV.TGBp1 + DI-P	100%(6/6)	50(3/6)
TBSV.TGBp1m1 + DI-P	nd	0(0/3)
TBSV.TGBp1m3 + DI-P	50(3/6)	33(1/3)
TBSV.TGBp1m5 + DI-P	nd	0(0/3)
TBSV.TGBp1m8 + DI-P	100(6/6)	0(0/6)
TBSV.TGBp1m9 + DI-P	nd	0(0/6)
TBSV.TGBp1m12 + DI-P	100(6/6)	0(0/6)

[a] Percentage of GFP-transgenic plants in which PTGS was suppressed in systemic tissue following inoculation with TBSV.GFP, TBSV.TGBp1, and TBSV.TGBp1 mutants + DI-P at 25°C and 28°C. Proportions of plants showing suppression relative to the total number of plants inoculated are shown in parentheses.

DISCUSSION

Wild-type TGBp1 silencing suppression in GFP-transgenic *N. benthamiana* plants was found to be temperature sensitive using the TBSV plus DI-P vector system. Silencing suppression was observed in 100% of plants maintained at 25°C but in only 50% of plants maintained at 28°C. Thus, although the increased temperature did not completely eliminate suppressor activity, TGBp1 did not suppress VIGS at the wild-type level described in previous reports (Voinnet et al, 2000). The increased temperature may have had some effect on the stability of TGBp1 and thereby altered its ability to suppress PTGS. Another possibility is that the silencing pathway itself is temperature sensitive. In experiments using *Cymbidium* ringspot virus (CymRSV) it was demonstrated that silencing is attenuated at low temperatures (between 15°C and 24°C) and at high temperatures (27°C) silencing is enhanced (Szittya et al., 2003). In GFP-transgenic plants, GFP mRNA was abundant at low temperature but decreased at higher temperature (Szittya et al., 2003). Thus, it is possible that with increasing temperature the PTGS mechanism is amplified in such a way that the TGBp1 suppressor is less effective than at lower temperature.

Mutations m1, m5, m8, m9, m12, which were introduced throughout the TGBp1 coding sequence, eliminated suppressor activity at 28°C. No specific sequence contributing to TGBp1 suppressor activity was identified by these mutations. The m12 mutation is in the ATPase active site; however, its effect on TGBp1 resembles the effects of mutations outside the ATPase active site. This would suggest that ATPase activity is not required for the suppression of silencing; however, further experiments are needed to determine if any of these mutations affect ATPase activity. It is possible that TGBp1 ATPase activity was equally affected by m1, m5, m8, m9, and m12 and that the ATPase

82

activity of TGBp1 is important for suppressor activity. Another possibility is that these mutations had a general effect on protein folding and thereby inactivated TGBp1 silencing suppressor activity. Since m8 and m12 do not affect TGBp1 silencing suppression at 25°C but do affect this activity at 28°C, it is possible that the mutations could confer greater temperature sensitivity to the TGBp1 protein.

The mutation m3 had reduced TGBp1 suppressor activity at 25°C and at 28°C. This mutation lies in a helicase/ATPase active site motif and might affect ATPase activity at both temperatures. The m3 mutation might disrupt TGBp1 suppressor activity by disrupting a sequence specifically required for suppressor activity or by affecting protein folding at both temperatures.

Previous studies have shown that TGBp1 is a silencing suppressor which blocks movement of the silencing signal to upper non-inoculated leaves. The results of this study did not identify a single "suppressor" domain within TGBp1 but suggest that there may be more than one domain of TGBp1 contributing to suppressor activity. Evidence that TGBp1 suppressor activity is temperature sensitive has not been presented previously. Further experiments are needed to determine the role temperature might play in silencing or TGBp1 suppression of silencing.

LITERATURE CITED

Anandalakshmi, R., Pruss, G., Ge, X., Marathe, R., Mallory, A. C., Smith, T. H., and Vance, V. B. A viral suppressor of gene silencing in plants. PNAS **95**(22):13079-13084.

Angell, S. M. and Baulcombe, D. C. (1995). Cell-to-cell movement of *Potato Virus X* revealed by microinjection of a viral vector tagged with the β-glucuronidase gene. The Plant J. 7(1): 135-140.

Angell, S. M., Davies, C., and Baulcombe, D.C. (1996). Cell-to-cell movement of *Potato Virus X* is associated with a change in the size-exclusion limit of plasmodesmata in trichome cells of Nicotiana clevelandii. Virology **216**: 197-201.

Angell, S. M. and Baulcombe, D. C. (1997). Consistent gene silencing in transgenic plants expressing a replicating *potato virus X* RNA. EMBO **16**: 3675-3684.

Baulcombe, D. C., Chapman, S., and Santa Cruz, S. (1995). Jellyfish green fluorescent protein as a reporter for virus infections. Plant J. 7(6): 1045-1053.

Baulcombe, D. C. (1996). RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Mol. Biol. **32**: 79-88.

Baulcombe, D. C. (2001). RNA silencing. Current Biology **12**: RB2-RB4.

Beck, D. L., Guilford, P.J., Voot, D.M., Andersen, M.T., and Forster, R.L.S. (1991). Triple gene block proteins of *White clover mosaic Potexvirus* are required for transport. Virology **183**: 695-702.

Bleykasten, C., Gilmer, D., Guilley, H., Richards, K.E., and Jonard, G. (1996). *Beet necrotic yellow vein virus* 42 kDa triple gene block protein binds nucleic acids in vitro. J. Gen. Virol. **77**: 889-897.

Brigneti, G., Voinnet, O., Li, W.-X., Ji, L.-H., Ding, S.-W., and Baulcombe, D.C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. EMBO **17**: 6739-46.

Carrington, J. C., Kasschau, K.D., Mahajan, S.K., and Schaad, M.C. (1996). Cell-to-cell and long-distance transport of viruses in plants. Plant Cell **8**: 1669-81.

Carrington, J. C., Jensen, P.E., and Schaad, M.C. (1998). Genetic evidence for an essential role for *Potyvirus* CI protein in cell-to-cell movement. Plant J. **14**: 393-400.

Chapman, S., Hills, G., Watts, J., and Baulcombe, D.C. (1992). Mutational analysis of the coat protein gene of *Potato virus X*: Effects on virion morphology and viral pathogenicity. Virology **191**: 223-230.

Citovsky, V., Zupan, J., Warnick, D., and Zambryski, P. (1992). Nuclear localization of Agrobacterium VirE2 protein in plant cells. Science **256**: 1802-1805.

Crawford, K. M., and Zambryski, P. C. (2001). Non-targeted and targeted protein movement through plasmodesmata in leaves in different developmental and physiological states. Plant Physiol. **125**(4):1802-1812.

Davenport, G. F. and Baulcombe, D. C. (1997). Mutation of the GKS motif of the RNA-dependent RNA polymerase from potato virus X disables of eliminates virus replication. J. Gen. Virol. **78**:1247-1251.

Deom, C. M., Schubert, K.R., Wolf, S., Holt, C.A., Lucas, W.J., and Beachy, R.N. (1990). Molecular characterization and biological function of the movement protein of *Tobacco mosaic virus* in transgenic plants. Proc. Nat. Acad. Sci., USA **87**: 3284-3288.

Davies, C., Hills, G., and Baulcombe, D. C. (1993). Sub-cellular localization of the 25-kDa protein encoded in the triple gene block of *Potato virus X*. Virology **197**(1): 166-175.

Deom, M. C., Lapidot, M., and Beachy, R.N. (1992). Plant virus movement proteins. Cell **69**: 221-224.

Ding, B., Haudenshield, J.S., Hull, R.J., Wolf, S., Beachy, R.N., and Lucas, W.J. (1992). Secondary plasmodesmata are specific sites of localization of the *Tobacco mosaic virus* movement protein in transgenic tobacco plants. Plant Cell **4**: 915-928.

Ding, B., Li, Q., Nguyen, L., Palukaitis, P., and Lucas, W.J. (1995). *Cucumber mosaic virus* 3a protein potentiates cell-to-cell trafficking of CMV RNA in tobacco plants. Virology **207**: 345-353.

Donald, R. G. K., Lawrence, D.M., and Jackson, A. O. (1997). The *Barley stripe mosaic virus* 58-Kilodalton Bb protein is a multifunctional RNA binding protein. J. Virol. **71**: 1538-1546.

Dougherty, W. G., Lindbo, J.A., Smith, H.A., Parks, T.D., Swaney, S., and Proebsting, W.M. (1994). RNA-mediated virus resistance in transgenic plants: Exploitation of a cellular pathway possibly involved in RNA degradation. Mol. Plant-Microbe Interact. **7**: 544-552.

Eagles, R. M., Balmori-Melian, E., Beck, D.L., Gardner, R.C. and Forster, R.L.S. (1994). Characterization of NTPase, RNA-binding and RNA helicase activities of the cytoplasmic inclusion protein of *Tamarillo mosaic potyvirus*. Eur. J. Biochem. **224**: 677-684.

Forster, R. L. S., Beck, D.L., Guilford, P.J., Voot, D.M., Van Dolleweerd, C.J., and Andersen M.T. (1992). The coat protein of *White clover mosaic potexvirus* has a role in facilitating cell-to-cell transport in plants. Virology **191**: 480-484.

Fridborg, I., Grainger, J., Page, A., Coleman, M., Findlay, K., Angell, S. (2003). TIP, a novel host factor linking callose degradation with the cell-to-cell movement of *Potato virus X*. Mol Plant- Microbe Interact **16**: 132-140.

Fujiwara, T., Giesman-Cookmeyer, D., Ding,B., Lommel, S., and Lucas, W.J. (1993). Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the *Red clover necrotic mosaic virus* movement protein. Plant Cell **5**: 1783-1794.

Heinlein, M., Padgett, H.S., Gens, J.S., Pickard, B.G., Casper, S.J., Epel, B.L., Beachy, R.N. (1998). Changing patterns of localization of the Tobacco mosaic virus movement protein and replicase to the endoplasmic reticulum and microtubules during infection. Plant Cell **10**: 1107-1120.

Hou, H. and Qiu, W. (2003). A novel co-delivery system consisting of a *Tomato bushy stunt virus* and a defective interfering RNA for studying gene silencing. J. Virol. Meths. **111**: 37-42.

Huisman, M. J., Linthorst, H.J.M., Bol, J.F., Cornelissen, B.J.C. (1988). The complete nucleotide sequence of *Potato virus X* and its homologies at the amino acid level with various plus-stranded RNA viruses. J. Gen. Virol. **69**: 1789-1798.

Imlau, A., Truernit, E., and Sauer, N. (1999). Cell-to-cell and long distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink tissues. Plant Cell **11**: 309-322.

Itaya, A., Hickman, H., Bao, Y., Nelson, R., and Ding, B. (1997). Cell-to-cell trafficking of Cucumber mosaic virus movement protein: Green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. Plant J. **12**: 1223-1230.

Itaya, A., Woo, Y.-M., Masuta, C., Bao, Y., Nelson, R. S., and Ding, B. (1998). Developmental regulation of intercellular protein trafficking through plasmodesmata in tobacco leaf epidermis. Plant Physiol. **118**: 373-385.

Itaya, A., Liang, G., Woo, Y.-M., Nelson, R. S., Ding, B. (2000). Nonspecific intercellular protein trafficking probed by green-fluorescent protein in plants. Protoplasma **213**: 165-175.

Kalinina, N. O., Samuilova, O.V., Fedorkin, O.N., Zelenina, D.A., Morozov, S.Y. (1998). Biochemical characterization and subcellular localization of a 25K transort protein of *Potato virus X*. Molec. Biol. **32**: 569-573.

Kasteel, D. T. J., Wellink, J., Goldbach, R.W., van Lent, J.W.M. (1997). Isolation and characterization of tubular structures of *Cowpea mosaic virus*. J. Gen. Virol. **78**: 3167-3170.

Klein, P. G., Klein, R.R., Rodriguez-Cerezo, E., Hunt, A.G. and Shaw, J.G. (1994). Mutational analysis of the *Tobacco vein mottling virus* genome. Virology **204:** 759-769.

Koonin, E. V., and Dolja, V.V. (1993). Evolution and taxonomy of positive-strand RNA viruses: Implications of comparative analysis of amino acid sequences. Biochem. Mol. Biol. **28**: 375-430.

Krishnamurthy, K., Mitra, R., Payton, M. E., and Verchot-Lubicz, J. (2002). Cell-to-cell movement of the *Potato virus X* 12K, 8K, or coat proteins may depend on the host, leaf developmental stage, and the PVX 25K protein. Virology **300**: 269-281.

Krishnamurthy, K., Heppler, M., Mitra, R., Blancaflor, E., Payton, M. E., Nelson, R. S. and Verchot-Lubicz, J. (2003). The *Potato virus X* TGBp3 protein associates with the ER network for virus cell-to-cell movement. Virology. **309**: 135-151.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W.M., and Dougherty, W.G. (1993). Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. Plant Cell **5**: 1749-1759.

Lough, T. J., Shash, K., Xoconostle-Cazares, B., Hofstra, K.R., Beck, D.L., Balmori, E., Forster, R.L.S., Lucas, W.J. (1998). Molecular dissection of the mechanism by which potexvirus triple gene block proteins mediate cell-to-cell transport of infectious RNA. Mol. Plant-Microbe Interact. **11**: 801-814.

Lough, T. J., Netzler, N. E., Emerson, S. J., Sutherland, P., Carr, F., Beck, D. L., Lucas, W. J., and Forster, R. L. (2000). Cell-to-cell movement of potexviruses: evidence for a ribonucleoprotein complex involving the coat protein and first triple gene block protein. Mol. Plant-Microbe Interact. **13**(9): 962-974.

Lucas, W. J., Ding, B., and van der Schoot, C. (1993). Plasmodesmata and the supracellular nature of plants. New Phytol. **125**: 435-476.

McLean, B. G., Zupan, J., and Zambryski, P.C. (1995). *Tobacco mosaic virus* movement protein associates with the cytoskeleton in tobacco cells. Plant Cell **7**, 2101-2114.
Melcher, U. (1990). Similarities between putative transport proteins of plant viruses. J. Gen. Virol. **71**: 1009-1018.

Melcher, U. (1990). Similarities between putative transport proteins of plant viruses. J. Gen. Virol. **71**: 1009-1018.

Melcher, U. (2000). The '30K' superfamily of viral movement proteins. J. Gen. Virol. **81**: 257-266.

Memelink, J., van der Vlugt, C. I. M., Linthorst, H. J. M., Derk, A. F. L. M., Asjes, C. J., and Bol, J. F. (1990). Homologies between the genomes of a carlavirus (Lily symptomless virus) and a potexvirus (*Lily virus X*) from lily plants. J. Gen. Virol. **71**: 917-924.

Mitra, R., Krishnamurthy, K., Blancaflor, E., Payton, M., and Nelson, R. S. and Verchot-Lubicz, J. (2003). The *Potato virus X* TGBp2 protein association with the endoplasmic reticulum plays a role in but is not sufficient for viral cell-to-cell movement. Virology **312**(1): 35-48.

Morozov, S. Y., Lukasheva, L. I., Chernov, B. K., Skryabin, K. G., and Atabekov, J. G. (1987). Nucleotide sequence of the open reading frames adjacent to the coat protein cistron in Potato virus X genome. FEBS Lett. **213,** 438-442.

Morozov et al., S. Y., Dolja, V.V., and Atabekov, J.G. (1989). Probable reassortment of genomic elements among elongated RNA-containing plant viruses. J. Mol. Evol. **29**: 52-62.

Morozov, S. Y., Fedorkin, O. N., Juttner, G., Schiemann, J., Baulcombe, D. C. and Atabekov, J. G. (1997). Complementation of a *Potato virus X* mutant mediated by bombardment of plant tissues with cloned viral movement protein genes. J. Gen. Virol. **78**: 2077-2083.

Morozov, S. Y., Solovyev, A.G., Kalinina, N.O., Fedorkin, O.N., Samuilova, O.V., Schiemann, J., and Atabekov, J.G. (1999). Evidence for two nonoverlapping functional domains in the *Potato virus X* 25K movement protein. Virology **260**: 55-63.

Morozov, S. Y. and Solovyev, A.G. (2003). Triple gene block: modular design of a multifunctional machine for plant virus movement. J. Gen. Virol. 84: 1351-1366.

Oparka, K. J., Duckett, C. M., Prior, D. A. M., and Fisher, D. B. (1994). Real-time imaging of phloem unloading in the root tip of Arabidopsis. Plant J. **6**: 759-766.

Oparka, K. J., Roberts, A. G., Roberts, I. M., Prior, D. A. M., and Santa Cruz, S. (1996). Viral coat protein is targeted to, but does not gate plasmodesmata during cell-to-cell movement of Potato virus X. Plant J. **10**(5): 805-813.

Oparka, K. J., Roberts, A. G., Poevink, P., Santa Cruz, S., Roberts, I., Pradel, K. S., Imlau, A., Kotlizky, G., Sauer, N., and Epel, B. (1999). Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. Cell **97**: 743-754.

Osman, T. A. M., Hayes, R.J., and Buck, K.W. (1992). Cooperative binding of the *Red clover necrotic mosaic virus* movement protein to single-stranded nucleic acids. J. Gen. Virol. **73**: 223-227.

Plasterk, R. H. A. (2002). RNA Silencing: The Genome's Immune System. Science **296**: 1263-1265.

Qiu, W., Park, J.-W., and Scholthof, H.B. (2002). Tombusvirus P19 mediated suppression of virus-induced gene silencing is controlled by genetic and dosage features that influence pathogenecity. Mol. Plant-Microbe Interact. **15**: 269-280.

Ratcliff, F., Harrison, B.D., and Baulcombe, D.C (1997). A similarity between viral defense and gene silencing in plants. Science **276**: 1558-60.

Ratcliff, F. Martin-Hernandez, A. M., and Baulcombe, D.C. (2001). *Tobacco rattle virus* as a vector for analysis of gene function by silencing. Plant J. **25**: 237-245.

Reichel, C., and Beachy, R.N. (1998). *Tobacco mosaic virus* infection induces severe morphological changes of endoplasmic reticulum. Proc. Natl. Acad. Sci. USA **95**: 11169-74.

Robards, A. W., and Lucas, W.J. (1990). Plasmodesmata. Annual Review Plant Physiology and Plant Molecular Biology **41**: 369-419.

Roberts, A. G., Santa Cruz, S., Roberts, I. M., Prior, D. A. M., Turgeon, R., and Oparka, K. J. (1997). Phloem unloading in sink leaves of Nicotiana benthamiana: Comparison of a fluorescent solute with a fluorescent virus. The Plant Cell **9**: 1381-1396.

Rodriguez-Cerezo, E., Findlay, K., Shaw, J.G., Lomonossoff, G.P., Qiu, S.G., Linstead, P., Shanks, M. and Risco, C. (1997). The coat and cylindrical inclusion proteins of a potyvirus are associated with connections between plant cells. Virol. **236**: 296-306.

Rouleau, M., Smith, R.J., Bancroft, J.B., and Mackie, G.A. (1994). Purification, properties, and subcellular localization of *Foxtail mosaic potexvirus* 26-kDa protein. Virology **204**: 254-265.

Ruiz, M. T., Voinnet, O., and Baulcombe, D.C. (1998). Initiation and maintenance of virus-induced gene silencing. Plant Cell **10**: 937-946.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual, second ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Santa Cruz, S., Roberts, A. G., Prior, D. A. M., Chapman, S., and Oparka, K. J. (1998). Cell-to-cell and phloem mediated transport of *Potato virus X*: the role of virions. Plant Cell **10**: 495-510.

Scholthof, H. B., Morris, T. J., and Jackson, A. O. (1993). The capsid protein gene of *Tomato busy stunt virus* is dispensable for systemic movement and can be replaced for localized expression of foreign genes. Mol. Plant-Microbe Interact. **6**: 309-322.

Scholthof, H. B., Scholthof, K.-B. G., and Jackson, A. O. (1996). Plant virus gene vector for the transient expression of foreign proteins in plants. Annu. Rev. Phytopathol. **34**: 299-323.

Scholthof, H. B. (1999). Rapid delivery of foreign genes into plants by direct rub-inoculation with intact plasmid DNA of a *Tomato bushy stunt virus* gene vector. J. Virol. **73**(9): 7823-7829.

Skryabin, K. G., Morozov, S. Y., Kraev, A. S., Rozanov, M. N., Chernov, B. K., Lukasheva, L. I. and Atabekov, J. G. (1988). Conserved and variable elements in RNA genomes of potexviruses. FEBS **240**: 33-40.

Solovyev, A. G., Stroganova, T.A., Zamyantnin, A.A. Jr., Fedorkin, O.N., Schiemann, J., and Morozov, S.Y. (2000). Subcellular sorting of small membrane-associated triple gene block proteins: TGBp3-assistaed targeting of TGBp2. Virology **269**: 113-127.

Spillane, C., Verchot, J., Kavanagh, T. A., and Baulcombe, D. C. (1997). Concurrent suppression of virus replication and rescue of movement-defective virus in transgenic plants expressing the coat protein of *Potato virus X*. Virology **236**(1): 76-84.

Szittya, G., Silhavy, D., Molnar, A., Havelda, Z., Lovas, A., Lakatos, L., Banfalvi, Z., and Burgyan, J. (2003). Low temperature inhibits RNA silencing-mediated defense by the control of siRNA generation. EMBO. **22**(3): 633-640.

Tamai, A., and Meshi, T. (2001). Cell-to-cell movement of *Potato virus X*: The role of p12 and p8 encoded by the second and third open reading frames of the triple gene block. Molec. Plant Microbe Interact. **14**: 1158-67.

van Lent, J., Storms, M., van der Meer, F., Wellink, J., and Goldbach, R. (1991). Tubular structures involved in movement of *Cowpea mosaic virus* are also formed in infected cowpea protoplasts. J. Gen. Virol. **72**: 2615-2623.

Vaucheret, H., Beclin, C., Elmayan, T., Feuerback, F., Godon, C., Morel, J.-B., Mourrain, P., Palauqui, J.-C., and Vernhettes, S. (1998). Transgene-induced gene silencing in plants. Plant J. **16**: 651-659.

Verchot, J., and Carrington, J. C. (1995). Evidence that the potyvirus P1 proteinase functions in trans as an accessory factor for genome amplification. J. Virol. **69**: 3668-3674.

Verchot, J., Angell, S. M., and Baulcombe, D. C. (1998). In vivo translation of the triple gene block of *Potato virus X* (PVX) requires two mRNAs. J. Virology **72**: 8316-8320.

Voinnet, O., Pinto, Y.M., and Baulcombe, D.C. (1999). Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses in plants. Proc. Nat. Acad. Sci., USA **96**: 14147-14152.

Voinnet, O., and Lederer, C. (2000). A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana. Cell **103**: 157-167.

Waigmann, E., Lucas, W.J., Citovsky, V., and Zambryski, P. (1994). Direct functional assay for Tobacco mosaic virus cell-to-cell movement protein and identification of a domain involved in increasing plasmodesmal permeability. Proc. Natl., Acad. Sci. USA **91**: 1433-37.

Waterhouse, P. M., Graham, M.W., and Wang, M.-B (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Nat. Acad. Sci., USA **95**: 13959-13964.

Wellink, J., and van Kammen, A. (1989). Cell-to-cell transport of Cowpea mosaic virus requires both the 58K/48K proteins and the capsid proteins. J. Gen. Virol. **70**: 2279-2286.

Wolf, S. D., C.M., Beachy, R.N. and Lucas, W.J. (1989). Movement protein of Tobacco mosaic virus modifies plasmodesmatal size exclusion limit. Science **246**: 377-39.

Yang, Y., Ding, B., Baulcombe, D.C. and Verchot, J. (2000). Cell-to-cell movement of the 25K protein of *Potato virus X* is regulated by three other viral proteins. Molec. Plant Microbe Interact **13**: 599-605.

VITA ②

AMANDA R. HOWARD

Candidate for the Degree of

Master of Science

Thesis: CHARCTERIZATION OF *POTATO VIRUS X* (PVX) TGBp1: ROLE IN VIRUS CELL-TO-CELL MOVEMENT AND SUPPRESSION OF VIRUS INDUCED GENE SILENCING

Major Field: Plant Pathology

Biographical:

> Education: Graduated from Oklahoma State University, Stillwater, OK in August 2001. Completed the Requirements for the Master of Science degree in Plant Pathology at Oklahoma State University in December, 2003.

> Professional Memberships: American Society for Virology, Phi Kappa Phi