A STUDY OF VASCULAR MOVEMENT OF POTATO VIRUS X (PVX) TGBp1, TGBp2 AND CP USING THE COMMELINA YELLOW MOTTLE VIRUS PROMOTER AND GFP

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in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2007

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ACKNOWLEDGMENTS

I wish to express my sincere and heartfelt gratitude to my advisor, Dr Jeanmarie Verchot-Lubicz, for her timely assistant, constant enthusiasm, utmost patience, constructive criticism, valuable suggestion, keen interest and steering out of my difficulties arisen throughout the studies. I am greatly indebted to the members of my advisory committee Dr Hassan Melouk, Dr Michael Anderson and Dr John Damicone for their helpful suggestion and guidance. I wish to thank Dr Charlotte Ownby, Terry Colberg and Dr Susheng Tan of the Electron Microscope Laboratory for their support. I am immensely thankful to my laboratory colleagues Dr Ho Jong Ju, Dr ChangMing Ye, Dr Timmy Samuel and Devinka Bamunusinghe for their help and friendship. This is also a great opportunity to express my heartfelt gratitude to my wife Tizita M. Degefu and my lovely daughter Aida T. Asamenew for their love and patience throughout the course of the study. Last but not least Dr Kefyalew Desta, Zewdie Alemayehu, Dr Abate Degefu, Dr Fiseha Abebe and their family members deserve special thanks for their consistent moral and material support. Finally, “All things were made by him, and nothing was made without him. John 1:3”

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NOMENCLATURE/LIST OF SYMBOLS

AMV ……*Alfalfa mosaic virus*

BaMV ……*Bamboo mosaic virus*

BAP…….....6-Benzylaminopurine

BMV……..*Brome mosaic virus*

BSA……..…Bovine Serum Albumin

BSMV……..*Barley stripe mosaic virus*

CaMV……*Cauliflower mosaic virus*

CC…….……Companion cell

CMoV…… *Carrot mottle virus*

CMV……*Cucumber mosaic virus*

CoYMV……*Commelina yellow mottle virus*

CP……...……...Coat protein

CPMV……..*Cowpea mosaic virus*

CRSV…….*Carnation ringspot virus*

DAPI……4',6-diamino-phenylindole

DNA…….....…… Deoxyribonucleic Acid

EM……Electron Microscope

ER…….……Endoplasmic reticulum

GFP……..…Green Fluorescent Protein

IAA……..…..Indol-3-Acetic Acid

kDa…….kilo Daltons

MP…..……..Movement protein

nts ……..Nucleotides

ORF ……...Open reading frame

PBS……...Phosphate-buffered saline

PCV…….*Peanut clump virus*

PD …….Plasmodesmata

PMTV……...*Potato mop top virus*

PSLV…….*Poa semilatent virus*

PSTVd…….……... *Potato spindle tuber viroid*

PVX……*Potato virus X*

PVY…….……..…….*Potato virus Y*

RCNMV…….*Red clover necrotic mosaic virus*

RNA……..……...…….. Ribonucleic acid

RNP……...Ribonucleoprotein complex

S……..……Second

SE……..……..Sieve element

SEL…….Size exclusion limit

sgRNAs…….....Subgenomic RNAs

siRNA……Small interfering RNA

SqLCV……..……..*Squash leaf curl virus*

TEV ……*Tobacco etch virus*

TGB……...Triple gene block

TMV……*Tobacco mosaic virus*

TVMV……...……...……*Tobacco vein mottle virus*

WClMV……*White clover mosaic virus*

CHAPTER I

INTRODUCTION AND LITERATURES REVIEW

Importance of *Potato virus X* (PVX)

Potato virus X (PVX), one of the most common of all viruses infecting potato (*Solanum tuberosum*), and is distributed worldwide (Hefferon et al., 1997; Malcuit et al., 1999; Kagiwada et al., 2005). PVX infects more than 240 plant species in 16 plant families; however the majority of hosts are in the family *Solanaceae* (Purcifull & Edwardson, 1981). In the US, Hoyman (1964) reported 31.54% and 21.85% reductions in potato vine and tuber yields, respectively, due to PVX infection. There are reports of synergistic diseases caused by mixed infection of PVX with other plant viruses (Vance et al., 1995; Pruss et al., 1997). For example, when PVX and *Potato virus Y* (PVY) occur in mixed infections, plants show a more severe disease than plants infected with either virus alone. Potato plants infected with PVX and PVY are stunted; show severe mosaic symptoms on leaves, and tuber yields are reduced up to 70% (Vance, 1991). Similar synergistic diseases have been reported in plants infected with PVX and other viruses such as *Tobacco etch virus* (TEV) and *Tobacco vein mottle virus* (TVMV) (Vance et al., 1995; Pruss et al., 1997).

Symptom of Disease Caused by PVX

PVX occurs in various cultivated plants, such as potato, tomato, and tobacco (Kagiwada et al., 2005). Typical symptoms in susceptible potato cultivars include stunting, chlorotic veins, reduction in leaf size, mosaic pattern on the leaves, as well as necrotic lesions in the tubers (Hoyman, 1964; Koeing and Lesemann, 1989). Symptoms and severity of PVX varies with virus strains and potato varieties (Schultz, 1944; Teri et al., 1977; Moreira et al., 1980; Kagiwada et al., 2005). PVX has been called the "healthy potato virus" because many potato varieties infected with PVX are symptomless (O'Brien and Rich, 1976).

Characteristics of PVX

PVX is a monopartite positive strand RNA virus, belongs to the genus *Potexvirus*, and has a filamentous virion that is approximately 550 nm long and 11-18 nm wide (Stols et al., 1970; Verchot-Lubicz et al., 2007). The genomic RNA has a 5' m^7GpppG cap (Sonenberg et al., 1978) and a 3' poly (A) tail (Morozov et al., 1987). The genomic sequence, excluding the poly (A) tract, is 6435 nucleotides (nts) long with five open reading frames (ORFs; Fig.1)(Huisman et al., 1988; Skryabin et al., 1988).

The 5' ORF (nts 85-4453) encodes the viral replicase which is a 166 kDa protein (Fig. 1). The replicase ORF is followed by three overlapping ORFs which comprise the "triple gene block" (Fig. 1). The triple gene block is conserved among several virus genera and encodes proteins required for virus movement. These proteins are named TGBp1, TGBp2, and TGBp3 and have molecular weights of 25 kDa, 12 kDa, and 8 kDa, respectively (Davies et al., 1993). The 3' ORF (nts 5650-6361) encodes the 25 kDa viral

coat protein (CP) (Fig. 1) (Huisman et al., 1988 and Skryabin et al., 1988). The 5' and 3' ends of the viral genome contain nontranslated sequences of 84 and 76 nts respectively, which are involved in regulating virus replication and translation (Pinck, 1975; Huisman et al., 1988; Hull, 2002; Kim et al., 2002; Batten et al., 2003; Kwon et al., 2005; Kwon and Kim, 2006). The PVX replicase is translated directly from the genomic RNA, which is functionally monocistronic. Two major subgenomic RNA (sgRNA) species of approximately 2.1 and 0.9 kb in length are responsible for expression of the triple gene block and CP, respectively (Kim and Hemenway, 1997).

TGBp1 is derived from sgRNA1(Verchot et al., 1998). TGBp2 and TGBp3 are derived from sgRNA2 (Verchot et al., 1998). The viral CP is expressed from sgRNA3 (Morozov et al., 1990) (Fig. 1). The triple gene block and CP are required for virus cell-to-cell movement (Beck et al., 1991; Forster et al., 1992; Santa Cruz et al., 1998; Verchot et al., 1998).

Cell to Cell and Vascular Virus Movement

Systemic virus infection often begins in a single leaf epidermal or mesophyll cell where it first multiplies and then spreads to adjacent cells and finally spreads long distance through the vasculature to distal organs (Santa Cruz et al., 1998; Ryabov et al., 1999). Both cell to cell and long distance virus movement rely on one or more virus encoded movement proteins (MPs)(Carrington et al., 1996; Lucas and Wolf, 1999). There is a great diversity among plant viruses in terms of the number of MPs and their distinct roles in promoting virus movement. Since the pathways for cell to cell and vascular movements are also different the mechanism of virus vascular transport is likely

to be different from the mechanism of virus cell-to-cell movement (Petty and Jackson, 1990; Scholthof et al., 1993; Ryabov et al., 1999; Zhu et al., 2002). Some viruses encode factors required for viral vascular transport which do not play a role in virus cell-to-cell movement.

Plant RNA viruses whose genomes encode a single MP include TMV, *Red clover necrotic mosaic virus* (RCNMV), *Carnation ring spot virus* (CRSV) and *Carrot mottle virus* (CMoV) (Fujiwara et al., 1993; Nurkiyanova et al., 2001; Hull, 2002). These viral MPs bind viral RNA and target it to plasmodesmata for cell to cell movement. Viruses which require two proteins to move include *Cucumber mosaic virus* (CMV) and *Brome mosaic virus* (BMV). These viruses require the 3a and CP for local movement (Nagano et al., 2001; Itaya et al., 2002; Tamai et al., 2003) but require 3a and 2b (Karl-Heinz et al., 2000; Nagano et al., 2001; Choi et al., 2005) for long distance movement (Lucas et al., 1995; Shi et al., 2002; Natilla et al., 2006). Tombusviruses also encode two MPs named P22 and P19 (Scholthof et al., 1995; Qiu et al., 2002; Hsu et al., 2004). P22 is required for virus cell-to-cell movement and P19 is required for virus vascular movement. Plant DNA viruses such as ssDNA geminiviruses require a mechanism to exit the nucleus and to move across the plasmodesmata. For example, *Squash leaf curl virus* (SqLCV) encodes two MPs named BR1 and BL1. BR1 is nuclear shuttle protein for facilitating movement between the cytoplasm and nucleus during virus replication and BL1 facilitates the movement of the virus to adjacent cells (Pascal et al., 1993; Pascal et al., 1994; Ingham et al., 1995; Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996; Qin et al., 1998; Ward and Lazarowitz, 1999).

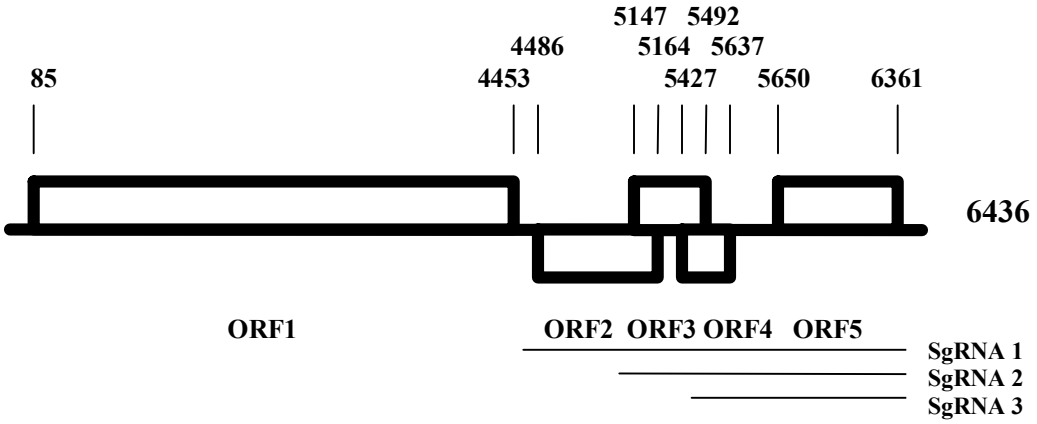


Figure. 1. Diagrammatic representation of the PVX genome. Boxes represent ORFs and the line represents genomic and three subgenomic (sgRNA) RNAs. The 5' and 3' nt positions of each ORF is indicated above the diagram. ORF1 encodes the 166 kDa viral replicase. ORF2, ORF3, and ORF4 are overlapping and comprise the triple gene block. ORF2 encodes the 25 kDa TGBp1, ORF3 encodes the 12 kDa TGBp2, and ORF4 encodes the 8 kDa TGBp3. ORF5 encodes the 25 kDa CP.

Viruses which require multiple genes for movement include members of the *Potyvirus* genus which rely on CP, HC-Pro, NIa, and CI for cell-to-cell and long-distance movement (Dolja et al., 1994; Cronin et al., 1995; Dolja et al., 1995; Kasschau et al., 1997; Schaad et al., 1997; Andersen and Johansen, 1998; Carrington et al., 1998; Kasschau and Carrington, 2001). Viruses belonging to the genera *Potex-, Carla-, Allexi-*, *Fovea-, Hordei-, Pomo-, Peclu-*, and *Benyvirus* contain an conserved block of overlapping ORFs called the triple gene block which encode three MPs (Solovyev et al., 1996; Wong et al., 1998; Liou et al., 2000; Solovyev et al., 2000; Lawrence and Jackson, 2001; Kalinina et al., 2002; Morozov and Solovyev, 2003; Koenig et al., 2004). These eight genera are divided into two groups (Lauber et al., 2005). The *Hordeivirus*-like group are rod-shaped viruses which rely solely on the triple gene block for cell-to-cell and vascular movement (Lawrence and Jackson, 2001; Kalinina et al., 2002; Morozov and Solovyev, 2003). The *Potexvirus*-like group are filamentous viruses which require CP in addition to the triple gene block to move (Beck et al., 1991; Forster et al., 1992; Baulcombe et al., 1995; Angell et al., 1996; Lough et al., 1998; Yang et al., 2000; Howard et al., 2004).

Virion formation (i.e. a complete viral particle) is required for the long-distance movement but not cell-to-cell movement of some viruses such as those belonging to the genera *Tobamovirus, Cucumovirus, and Dianthovirus* (Carrington et al., 1996; Prokhnevsky et al., 2002; Soto et al., 2005). Some viruses do not require virion formation for long distance movement. For example, *Potato mop-top virus* (*Pomovirus*) RNA can move systemically over a long distance without CP in a non virion form (McGeachy and Barker, 2000). *Potato spindle tuber viroid* (PSTVd) is a pathogenic

RNA that does not encode proteins and is not encapsidated, and yet it replicates autonomously and traffics systemically within an infected plant (Zhu et al., 2002). Potexviruses require CP for cell-to-cell and vascular transport (Lough et al., 1998) but it is not known if virion formation, per se, is required for long distance movement.

The systemic invasion of plants by viruses requires two steps. First, virus moves relatively at a slow pace from cell to cell until reaching the vascular tissues. Second, viruses move much faster through vascular system over a long-distance (Carrington et al., 1996; Gilbertson and Lucas, 1996; Ryabov et al., 1999). Three elements are necessary for successful cell-to-cell and vascular movement of viruses in plants: 1) a mobile form of the virus such as virion or ribonucleoprotein complex; 2) pre existing endogenous host cellular structures for macromolecular movement and 3) viral encoded MPs capable of interacting with mobile form of the virus, modifying pre-existing host cellular structures (plasmodesmata, ER, vesicles etc) and/or overcoming host defense mechanisms are crucial (Lucas et al., 1995; Carrington et al., 1996; Taiz and Zeiger, 2002; Ju et al., 2005).

It is generally accepted that viruses move from cell to cell through plasmodesmata by hijacking the mechanism that evolved for cell to cell communication (Fig. 2). Plasmodesmata are pores that exist between plant cells which expand and contract to allow selective movement of solutes and macromolecules between neighboring cells (McLean et al., 1997; Zambryski and Crawford, 2000; Cilia and Jackson, 2004; Faulkner et al., 2005). Virus encoded movement proteins (MPs) generally function to trigger expansion of plasmodesmata thereby allowing viral transport between adjacent cells (McLean et al., 1997; Oparka et al., 1999; Oparka and Turgeon, 1999; Oparka and Roberts, 2001; Krishnamurthy et al., 2002; Howard et al., 2004; Faulkner et al., 2005; Ju

et al., 2005). Some viruses move through plasmodesmata as intact virion particles while others move as ribonucleoprotein complexes (Citovsky and Zambryski, 1993; Lough et al., 1998; Lough et al., 2000; Krishnamurthy et al., 2002; Karpova et al., 2006).

There are at least two types of plasmodesmata modifications by viral MPs. Some virus encoded MPs are capable of dilating existing plasmodesmata to facilitate protein and nucleic acid movement (Barton, 2001). The typical example for this mechanism is the TMV MP which is capable of inducing a 10-fold increase in the size exclusion limit (SEL) of plasmodesmata (Wolf et al., 1989; Ding et al., 1992). Recent studies indicate that the TMV MP associates with viral replication complexes and transports them across plasmodesmata (Ding et al., 2004; Kawakami et al., 2004; Liu et al., 2005). The TMV replication complexes look like membrane vesicles containing viral replicase and RNA (Kawakami et al., 2004). The TMV MP associates with these replication vesicles, triggers expansion of the plasmodesmata, and then carries the vesicles into adjacent cells (Kawakami et al., 2004). The second type of plasmodesmata modification is the one adopted by icosahedral viruses. For example, *Cowpea mosaic virus* (CPMV), *Alfalfa mosaic virus* (AMV) and *Brome mosaic virus* (BMV) have icosahedral particles that move from cell to cell with the aid of the viral MPs (Kasteel et al., 1993; Bertens et al., 2003). CPMV encodes two MPs which form tubular structures which extend across plasmodesmata and serve as conduits for transferring virion between cells.

Functions of PVX Movement Proteins in Cell-to-Cell and Vascular Transport

While the role of PVX TGB and CP proteins in virus cell-to-cell movement have been described (Lough et al, 1998) there is very little known about the role of these proteins in virus vascular transport (Verchot, 2005; Howard et al., 2004; Mitra et al., 2003; Krishnamurthy et al., 2002; Verchot et al., 1998; Santa Cruz et al., 1998 and Angell, 1996). The PVX TGBp1 protein is multifunctional. TGBp1 has RNA helicase activity, expands plasmodesmata SEL to promote virus cell-to-cell trafficking, and suppresses RNA silencing (Verchot 2005; Howard et al., 2004; Yang et al., 2000; Morozov et al., 1999). Mutations disabling the TGBp1 protein helicase activity also eliminate the ability of the TGBp1 protein to expand the plasmodesmata (Howard et al., 2004; Kalinina et al., 2002). The TGBp2 and TGBp3 proteins are membrane binding proteins (Haupt et al., 2005; Verchot, 2005; Schepetilnikov et al., 2005; Morozov & Solovyev, 2003). The potexvirus TGBp2 protein has two membrane-spanning domains while the TGBp3 protein has one membrane-spanning domain (Mitra et al., 2003; Krishnamurthy et al., 2003). The potexvirus TGBp2 and TGBp3 proteins were shown to be associated with the endoplasmic reticulum (ER) during virus infection (Fedorkin et al., 2000). Mutations within the transmembrane domains of TGBp2 and TGBp3 resulted in elimination of PVX cell-to-cell movement (Krishnamurthy et al. 2002; Mitra et al., 2003; Ju et al., 2005, Ju et al., 2007).

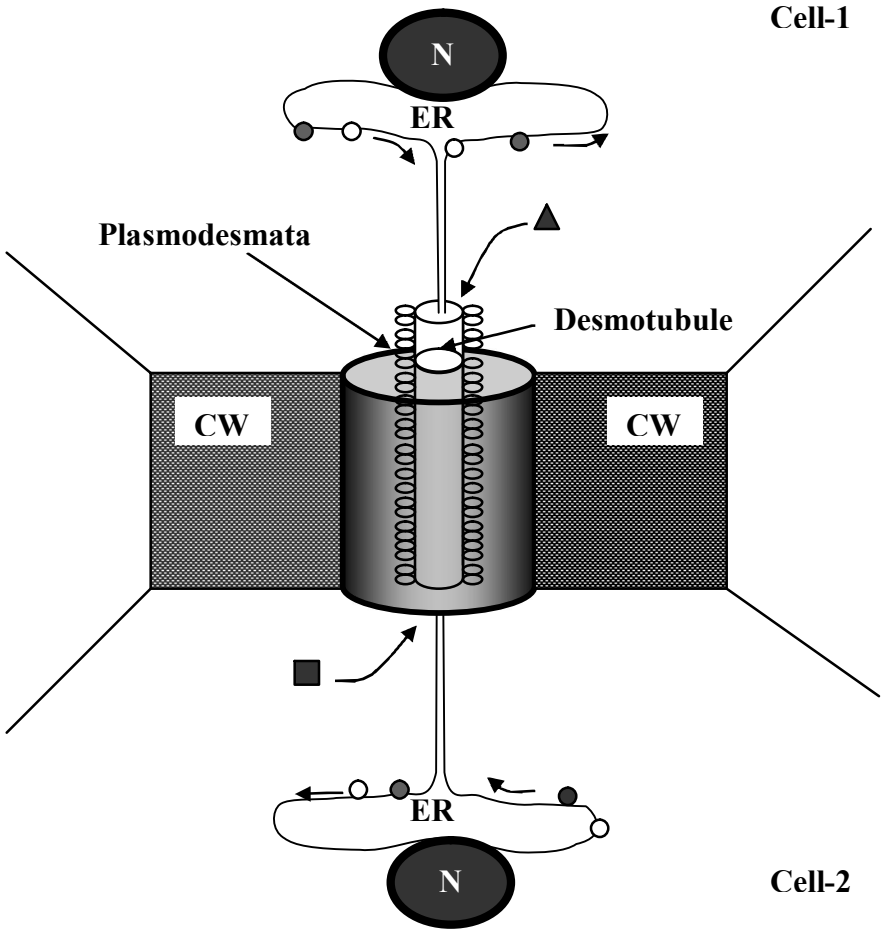


Figure 2. Diagrammatic representation of plasmodesmata. Two adjacent cells (Cell-1 and Cell-2) are connected by a plasmodesmata pore (Zambryski and Crawford, 2000). ER is continuous between these two cells and ER strand is running through desmotubule. On either side of the desmotubles are aggregate of plasmodesmata proteins. These proteins might be actins and myosin and may play roles in plasmodesmata opening and closing (Barton, 2001). Molecules can move from cell to cell along ER strand or using other cytoskeletons. Dark circles and white circles represent those molecules that move along ER and the dark triangle and box indicate those molecules passing through plasmodesmata using other cytoskeleton. Arrows along ER indicate the direction of movement. CW, Cell wall; N, Nucleus.

Phloem Structure and Functions

Although phloem has been studied and long been known for long distance translocation and distribution of photoassimilates and nutrients, recent research has shown that proteins, small interfering RNAs (siRNAs), phytohormones, plant viruses and bacteria are also transported through phloem (Nelson and van Bel, 1998; Lucas and Wolf, 1999; Oparka and Turgeon, 1999). More than 90% of photoassimilates that contribute for plant biomass are transported, principally in the form of sugars and amino nitrogen compounds, from source to sink plant parts through phloem (Lalonde et al., 2003).

The three major components of phloem (Fig.3) include companion cells, sieve elements and phloem parenchyma (Esau, 1965, , 1973; Evert, 1982). The companion cells and sieve elements originated from the same precursor cells (Esau 1965). Ninety percent of the proteins and nucleic acids that enter the sieve elements are synthesized in the companion cells and pass through plasmodesmata connections. Companion cells also play a significant role in loading sugars into the sieve elements (Wooding, 1967). Companion cells typically have larger numbers of ribosome and mitochondria which make them more metabolically active than other plant cells. The mature enucleated sieve elements (SEs) retain only a highly degenerated cytoplasm, but they can remain viable and functional for decades (Parthasarathy and Larry, 1976; Oparka and Turgeon, 1999). This is because all of the cellular functions of a SE element are carried out by the companion cell (Wooding, 1967).

Young sieve cells have the same organelles as mesophyll cells and other cells but as sieve cells undergo significant modifications that include highly selective autolysis of several organelles and a process of selective degradation resulting in the mature

enucleated sieve elements (Esau, 1965; Sjolund, 1997; Oparka and Turgeon, 1999). The entire sieve element protoplast, the central vacuole, cytoplasmic ribosome, Golgi bodies, and nucleus are selectively degraded and eventually eliminated (Oparka and Turgeon, 1999). Mature sieve elements retain certain organelles including filamentous phloem proteins (P-proteins), stacked cisternal aggregates of ER concentrated at the lateral walls, plasma membrane, modified mitochondria, plastids and few vacuoles (Fig. 4) (Cronshaw and Esau, 1967).

Sieve elements are approximately 20–30 μm in diameter and 100–500 μm in length and about 20–100 sieve elements per cm are aligned end to end to form a continuous tube that conducts macromolecules throughout the plant (Henton et al., 2002). Plasmodesmata connecting sieve cells across the end walls become greatly modified to form the sieve-plate pores during sieve element maturation (Oparka and Turgeon, 1999). The cytoplasm of a companion cell is connected to the sieve element with numerous plasmodesmata to form companion cell-sieve element complex by plasmodesmata (Esau, 1973; Evert, 1982; Taiz and Zeiger, 2002).

Mechanisms of Phloem Loading and Unloading

Phloem loading refers to the movement of photosynthates from the mesophyll to the sieve element /companion cells (SE/CC)-complex (Hendrix, 1977; Giaquinta, 1979; Bennett et al., 1984; Turgeon and Wimmers, 1988; van Kesteren et al., 1988; Turgeon and Beebe, 1991; Stadler et al., 1995). Phloem loading is an energy-requiring process that elevates the solute content of sieve elements and companion cells to levels far above those of surrounding cells (Kuhn et al., 1997; Lalonde et al., 2003). The pressure

gradient between source and sink organs drives long-distance transport (Fig 4)(Donald, 1978; Voitsekhovskaja et al., 2006). Two modes of phloem loading are symplastic or apoplastic (Van Bel, 1993; Santa Cruz, 1999; Oparka and Santa Cruz, 2000; Lalonde et al., 2003). When solutes enter the CC- SE complex via the plasma membrane, the SE loading is termed apoplastic. Phloem loading is denoted apoplastic if plasmodesmata are virtually absent at some point in the pathway irrespective of the site of symplastic discontinuity. The transport assimilates from the SE to the recipient sink cells refer to phloem unloading (Patrick, 1997; Oparka and Santa Cruz, 2000).

Phloem Specific Promoters

Research in this proposal will use the *Commelina yellow mosaic virus* (CoYMV) promoter to express viral proteins in phloem tissues (Medberry et al., 1992; Medberry and Olszewski, 1993; Matsuda et al., 2002). CoYMV is a DNA virus that is restricted to infecting phloem tissue. The CoYMV promoter used in this study was isolated by Neil Olszewski and was shown to express specifically in phloem CCs in transgenic tobacco plants. Comparison of CaMV 35S promoter and the CoYMV promoter in stably transformed maize callus showed that these two promoters have similar strengths (Medberry et al., 1992; Medberry and Olszewski, 1993) making the CoYMV promoter a good choice for expressing foreign genes in the phloem of transgenic plants. A CoYMV promoter fragment was isolated from the full CoYMV genomic clone pCoYMV89 (Medberry et al., 1990). The promoter fragment encompasses the region from -1026 to +12 relative to the 5' end of the CoYMV transcript. Promoter sequences contributing

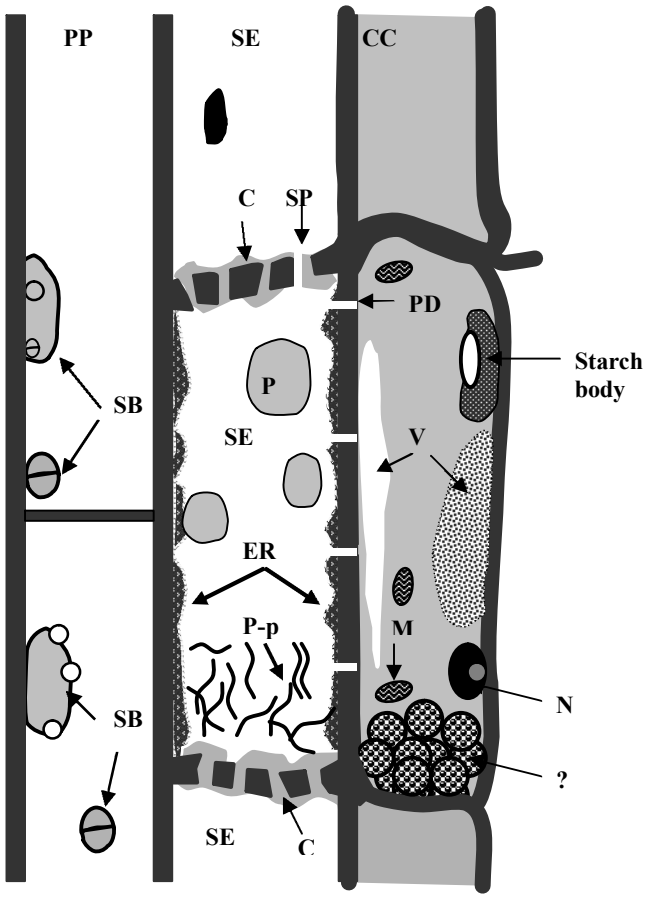


Figure 3. Phloem anatomy seen in longitudinal section under the electron microscope (Esau, 1965; Taiz and Zeiger, 2002; Van Bel, 2003). Companion cells (CC) are filled with gray color, sieve elements (SE) and phloem parenchyma (PP) is indicated. Organelles found in CCs but not SE are shown: mitochondria (M), nucleus, (N), few vacuoles are indicated in white (V), starch bodies, and the "?" highlights clusters of mitochondria, chloroplast and electron dense material. SEs are connected to CC by plasmodesmata (PD). The junction between two adjacent SEs is called a sieve plate (SP). Callose deposited around the SP is indicated in gray. SE contains filamentous phloem proteins (P-p), plastids (P) and stacked cisternal aggregates of endoplasmic reticulum (ER). Phloem parenchyma also contains starch bodies (SB) of various shapes and sizes.

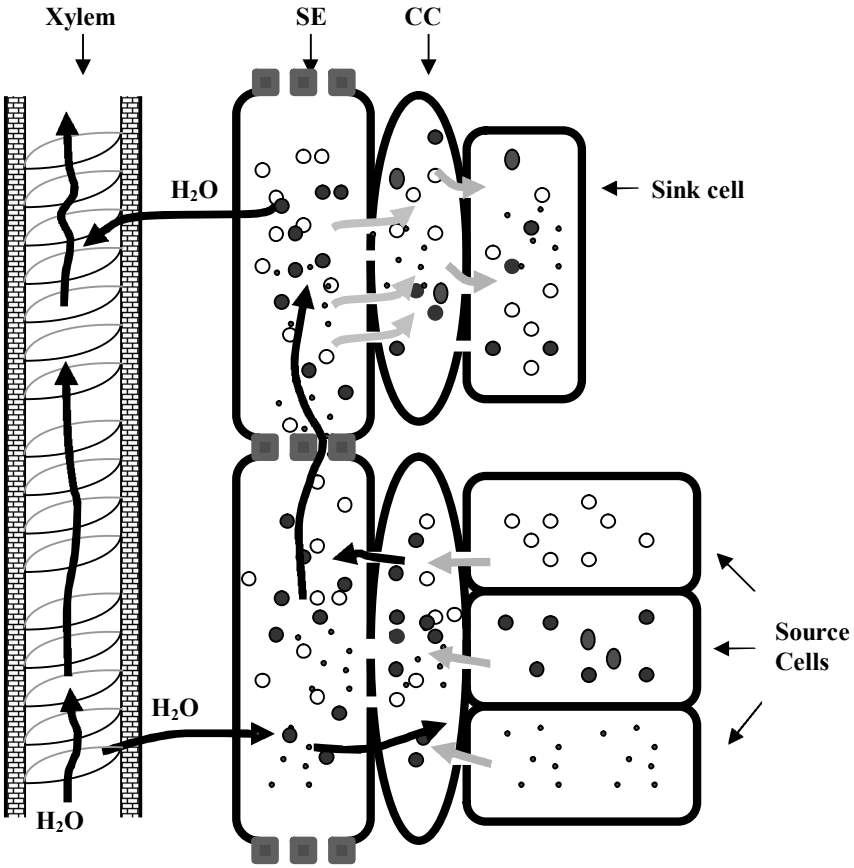


Figure 4. Diagrammatic representation of pressure flow model for phloem translocation and unloading during source to sink transition (Evert, 1982; Taiz and Zeiger, 2002; Voitsekhovskaja et al., 2006). The pressure-flow hypothesis describes the movement of solutes, such as sucrose, oligosaccharides, sugar alcohols, electrolytes, amino acids, and certain organic and inorganic acids molecules through the phloem (Thompson and Holbrook, 2003; Voitsekhovskaja et al., 2006). The pressure gradient between source and sink organs drives long-distance transport of various molecules, indicated here as black and white spheres (•●○). Since most plant viruses move long distance through the phloem, a model of virus movement was proposed based on the pressure flow

hypothesis to explain virus source-sink translocation (Robert et al., 1999). However, it is still unclear whether it is a pressure flow in bulk that is driving viruses into the CC-SE complex for long distance movement into upper leaves. For the pressure flow model, solutes enter to the CC- SE complex by active transport in source cells. This leads to build–up of turgor pressure in CC-SE complex to drive solutes upward along a concentration gradient in SE and then solutes unload in sink leaves. Black arrows show the direction of water flow and grey arrows show the direction solutes flow from source cells to sink cells.

specifically to vascular specific gene expression were found by deletion analysis to be located between nt positions -159 and -84 (Medberry and Olszewski, 1993).

The CoYMV promoter has advantages over the use of endogenous plant promoters because it is expressed as plants develop. Studies on a number of endogenous plant promoters suggest that CCs from different organs at different developmental stages of a given plant have unique regulatory patterns of gene expression (Matsuda et al., 2002). For example, the *Arabidopsis thaliana* sucrose transporter-2 (AtSUC2) promoter is commonly used for expression of foreign genes in the phloem. However, the AtSUC2 promoter is active predominantly in the CCs of mature source leaves (Elisabeth and Norbert, 1995; Imlau et al., 1999; Oparka et al., 1999) and cannot be used to study gene expression in young tissues. Another example is the galactinol synthase (GAS) promoter which is active in the CCs of minor veins in mature *Arabidopsis* and tobacco leaves (Haritatos et al., 2000). Thus, the GAS promoter cannot be used to study gene expression in other types of veins.

Research Objectives

Studies have shown that the requirements for long distance transport of PVX through the vascular system might differ from the requirements for virus cell-to-cell movement (Spillane et al., 1997; Santa Cruz et al., 1998). In this study we conducted experiments to determine the role for the PVX TGBp1, PVX TGBp2 and CP proteins in virus long distance movement. This study has the following objectives:

Objective 1: Prepare transgenic plants expressing TGBp1, TGBp2, CP in companion cells using the CoYMV promoter.

Objective 2: determine if fusion proteins move out of the phloem into mesophyll cells:

Objective 3: determine if the fusion proteins move through phloem sieve tubes.

Objective 4: determine if PVX can infect transgenic plants and if fusion proteins move along with PVX through the phloem and exit into mesophyll tissues.

LITERATURES CITED

Andersen K, Johansen IE (1998) A single conserved amino acid in the coat protein gene of *Pea seed-borne mosaic potyvirus* modulates the ability of the virus to move systemically in *Chenopodium quinoa*. Virology **241:** 304-311

Barton MK (2001) Giving meaning to movement. Cell **107:** 129-132

Batten JS, Yoshinari S, Hemenway C (2003) *Potato virus X*: a model system for virus replication, movement and gene expression. Molec. Plant Pathol. **4:** 125-131

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

Beck DL, Guilford PJ, Voot DM, Andersen MT, Forster RLS (1991) Triple gene block proteins of *White clover mosaic potexvirus* are required for transport. Virology **183:** 695-702

Bennett AB, Sweger BL, Spanswick RM (1984) Sink to source translocation in soybean. Plant Physiol **74:** 434-436

Bertens P, Heijne W, van der Wel N, Wellink J, Kammen AV (2003) Studies on the C-terminus of the *Cowpea mosaic virus* movement protein. Arch Virol **148:** 265-279

Carrington JC, Jensen PE, Schaad MC (1998) Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. Plant J **14:** 393-400

Carrington JC, Kasschau KD, Mahajan SK, Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell **8:** 1669-1681

Choi SK, Palukaitis P, Min BE, Lee MY, Choi JK, Ryu KH (2005) *Cucumber mosaic virus* 2a polymerase and 3a movement proteins independently affect both virus movement and the timing of symptom development in zucchini squash. J Gen Virol **86:** 1213-1222

Cilia ML, Jackson D (2004) Plasmodesmata form and function. Curr Opin Cell Biol **16:** 500-506

Citovsky V, Zambryski P (1993) Transport of nucleic acids through membrane channels: snaking through small holes. Ann Rev Microbiol **47:** 167-197

Cronin S, Verchot J, Haldeman-Cahill R, Schaad MC, Carrington JC (1995) Long-distance movement factor: a transport function of the potyvirus helper component proteinase. Plant Cell **7:** 549-559

Cronshaw J, Esau K (1967) Tubular and fibrillar components of mature and differentiating sieve elements. J. Cell Biol. **34:** 801-815

Ding B, Haudenshield JS, Hull RJ, Wolf S, Beachy RN, Lucas WJ (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 XS, Liu J, Cheng NH, Folimonov A, Hou YM, Bao Y, Katagi C, Carter SA, Nelson RS (2004) The *Tobacco mosaic virus* 126-kDa protein associated with virus replication and movement suppresses RNA silencing. Mol Plant Microbe Interact **17:** 583-592

Dolja VV, Haldeman-Cahill R, Montgomery AE, Vandenbosch KA, Carrington JC (1995) Capsid protein determinants involved in cell-to-cell and long distance movement of *Tobacco etch potyvirus*. Virology **206:** 1007-1016

Dolja VV, Haldeman R, Robertson NL, Dougherty WG, Carrington JC (1994) Distinct functions of capsid protein in assembly and movement of *Tobacco etch potyvirus* in plants. Embo J **13:** 1482-1491

Donald BF (1978) An evaluation of the Münch hypothesis for phloem transport in soybean. Planta **139:** 25-28

Elisabeth T, Norbert S (1995) The promoter of the *Arabidopsis thaliana* SUC2 sucrose-H+ symporter gene directs expression of β-glucuronidase to the phloem: Evidence for phloem loading and unloading by SUC2. Planta **196:** 564-570

Esau K (1965) Plant anatomy, Ed 2nd. John Wiley & Sons, Inc., New York

Esau K (1973) Comparative structure of companion cells and phloem parenchyma cells in *Mimosa pudica* L. Ann Bot **37:** 625-632

Evert RF (1982) Sieve-Tube structure in relation to function. BioScience **32:** 789-795

Faulkner C, Brandom J, Maule A, Oparka K (2005) Plasmodesmata 2004. Surfing the symplasm. Plant Physiol **137:** 607-610

Fedorkin ON, Merits A, Lucchesi J, Solovyev AG, Saarma M, Morozov SY, Makinen K (2000) Complementation of the movement-deficient mutations in *Potato virus X*: potyvirus coat protein mediates cell-to-cell trafficking of C-terminal truncation but not deletion mutant of potexvirus coat protein. Virology **270:** 31-42

Forster RLS, Beck DL, Guilford PJ, Voot DM, Van Dolleweerd CJ, Andersen MT (1992) Thecoat 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 SA, Lucas WJ (1993) Cell-to-cell trafficking of macromolecules through plasmodesmata potentiated by the *Red clover necrotic mosaic virus* movement protein. Plant Cell **5:** 1783-1794

Giaquinta RT (1979) Phloem loading of sucrose: Involvement of membrane ATPase and proton transport. Plant Physiol **63:** 744-748

Gilbertson RL, Lucas WJ (1996) How do viruses traffic on the 'vascular highway'? Trends Plant Sci **1:** 260-268

Haritatos E, Ayre BG, Turgeon R (2000) Identification of phloem involved in assimilate loading in leaves by the activity of the Galactinol Synthase promoter. Plant Physiol. **123:** 929-938

Hefferon KL, Doyle S, AbouHaidar MG (1997) Immunological detection of the 8K protein of *Potato virus X* (PVX) in cell walls of PVX-infected tobacco and transgenic potato. Arch Virol **142:** 425-433

Hendrix JE (1977) Phloem loading in squash. Plant Physiol **60:** 567-569

Henton SM, Greaves AJ, Piller GJ, Minchin PEH (2002) Revisiting the Munch pressure-flow hypothesis for long-distance transport of carbohydrates: modelling

the dynamics of solute transport inside a semipermeable tube. J. Exp. Bot. **53:** 1411-1419

Howard AR, Heppler ML, Ju HJ, Krishnamurthy K, Payton ME, Verchot-Lubicz J (2004) *Potato virus X* TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves. Virology **328:** 185-197

Huisman MJ, Linthorst HJ, Bol JF, Cornelissen JC (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

Hull RJ (2002) Matthews Plant Virology Ed 4th ed. Academic Press, San Diego, CA.

Imlau A, Truernit E, 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

Ingham DJ, Pascal E, Lazarowitz SG (1995) Both bipartite geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity. Virology **207:** 191-204

Itaya A, Ma F, Qi Y, Matsuda Y, Zhu Y, Liang G, Ding B (2002) Plasmodesma-mediated selective protein traffic between "symplasmically isolated" cells probed by a viral movement protein. Plant Cell **14:** 2071-2083

Ju HJ, Samuels TD, Wang YS, Blancaflor E, Payton M, Mitra R, Krishnamurthy K, Nelson RS, Verchot-Lubicz J (2005) The *Potato virus X* TGBp2 movement

protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol **138:** 1877-1895

Kagiwada S, Yamaji Y, Komatsu K, Takahashi S, Mori T, Hirata H, Suzuki M, Ugaki M, Namba S (2005) A single amino acid residue of RNA-dependent RNA polymerase in the *Potato virus X* genome determines the symptoms in Nicotiana plants. Virus Research **110:** 177-182

Kalinina NO, Rakitina DV, Solovyev AG, Schiemann J, Morozov SY (2002) RNA helicase activity of the plant virus movement proteins encoded by the first gene of the triple gene block. Virology **296:** 321-329

Karl-Heinz H, Carolin Z, Heinrich B (2000) RNA 2 of *Cucumber mosaic virus* subgroup I strain NT-CMV is involved in the induction of severe symptoms in tomato. Eur J Plant Pathol **106:** 95-99

Karpova OV, Zayakina OV, Arkhipenko MV, Sheval EV, Kiselyova OI, Poljakov VY, Yaminsky IV, Rodionova NP, Atabekov JG (2006) *Potato virus X* RNA-mediated assembly of single-tailed ternary 'coat protein-RNA-movement protein' complexes. J Gen Virol **87:** 2731-2740

Kasschau KD, Carrington JC (2001) Long-distance movement and replication maintenance functions correlate with silencing suppression activity of potyviral HC-Pro. Virology **285:** 71-81

Kasschau KD, Cronin S, Carrington JC (1997) Genome amplification and long-distance movement functions associated with the central domain of *Tobacco etch potyvirus* helper component-proteinase. Virology **228:** 251-262

Kasteel D, Wellink J, Verver J, van Lent J, Goldbach R, van Kammen A (1993) The involvement of *Cowpea mosaic virus* M RNA-encoded proteins in tubule formation. J Gen Virol **74:** 1721-1724

Kawakami S, Watanabe Y, Beachy RN (2004) *Tobacco mosaic virus* infection spreads cell to cell as intact replication complexes. P Natl Acad Sci USA **101:** 6291-6296

Kim KH, Hemenway C (1997) Mutations that alter a conserved element upstream of the *Potato virus* X triple block and coat protein genes affect subgenomic RNA accumulation. Virology **232:** 187-197

Kim KH, Kwon SJ, Hemenway C (2002) Cellular protein binds to sequences near the 5' terminus of *Potato virus X* RNA that are important for virus replication. Virology **301:** 305-312

Koenig R, Pleij CW, Loss S, Burgermeister W, Aust H, Schiemann J (2004) Molecular characterisation of potexviruses isolated from three different genera in the family Cactaceae. Arch Virol **149:** 903-914

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

Kuhn C, Franceschi VR, Schulz A, Lemoine R, Frommer WB (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. Science **275:** 1298-1300

Kwon SJ, Kim KH (2006) The SL1 stem-loop structure at the 5'-end of *Potato virus* X RNA is required for efficient binding to host proteins and for viral infectivity. Mol Cells **21:** 63-75

Kwon SJ, Park MR, Kim KW, Plante CA, Hemenway CL, Kim KH (2005) cis-Acting sequences required for coat protein binding and in vitro assembly of *Potato virus X*. Virology **334:** 83-97

Lalonde S, Tegeder M, Throne-Holst M, Frommer W, Patrick J (2003) Phloem loading and unloading of sugars and amino acids. Plant Cell Environ **26:** 37-56

Lalonde S, Weise A, Walsh RP, Ward JM, Frommer WBSl, M Segeder, M Throne-Holst, WB Frommer, JW Patrick (2003) Fusion to GFP blocks intercellular trafficking of the sucrose transporter SUT1 leading to accumulation in companion cells Phloem loading and unloading of sugars and amino acids. BMC Plant Biol **3:** 8

Lauber E, Jonard G, Richards K, Guilley H (2005) Nonregulated expression of TGBp3 of hordei-like viruses but not of potex-like viruses inhibits *Beet necrotic yellow vein virus* cell-to-cell movement. Arch Virol **150:** 1459-1467

Lawrence DM, Jackson AO (2001) Interactions of the TGB1 protein during cell-to-cell movement of *Barley stripe mosaic virus*. J Virol **75:** 8712-8723

Lin HX, White KA (2004) A complex network of RNA-RNA interactions controls subgenomic mRNA transcription in a tombusvirus. Embo J **23:** 3365-3374

Liou DY, Hsu YH, Wung CH, Wang WH, Lin NS, Chang BY (2000) Functional analyses and identification of two arginine residues essential to the ATP-utilizing activity of the triple gene block protein 1 of *Bamboo mosaic potexvirus.* Virology **277:** 336-344

Liu H, Boulton MI, Oparka KJ, Davies JW (2001) Interaction of the movement and coat proteins of *Maize streak virus*: implications for the transport of viral DNA. J Gen Virol **82:** 35-44

Liu J-Z, Blancaflor EB, Nelson RS (2005) The *Tobacco mosaic mirus* 126-Kilodalton protein, a constituent of the virus replication complex, alone or within the complex aligns with and traffics along microfilaments. Plant Physiol. **138:** 1853-1865

Lough T, Shash K, Xoconostle-Cazares B, Hofstra K, Beck D, Balmori E, Forster R, Lucas W (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 TJ, Netzler NA, Emerson SJ, Sutherland P, Carr F, Beck DL, Lucas WJ, Forster RL (2000) Cell-to-cell movement of potexviruses: evidence for a ribonucleic protein complex involving the coat protein and first triple gene block protein. Mol Plant Microbe Interact **13:** 13:962–974

Lucas W, Bouché-Pillon S, Jackson D, Nguyen N, Baker L, Ding B, Hake S (1995) Selective trafficking of KNOTTED1 homeodomain protein and its mRNA through plasmodesmata. Science **270:** 1980 -1983

Lucas WJ, Wolf S (1999) Connections between virus movement, macromolecular signaling and assimilate allocation. Curr Opin Plant Biol **2:** 192-197

Malcuit IM, Marano M, Kavanagh T, Jong W, Forsyth A, Baulcombe D (1999) The 25 kDa movement protein of PVX elicits Nb-mediated hypersensitive cell death in potato. Mol Plant-Microbe Interact **12:** 536–543

Matsuda Y, Liang G, Zhu Y, Ma F, Nelson RS, Ding B (2002) The *Commelina yellow mottle virus* promoter drives companion-cell-specific gene expression in multiple organs of transgenic tobacco. Protoplasma **220:** 51-58

McGeachy K, Barker H (2000) *Potato mop-top virus* RNA can move long distance in the absence of coat protein: evidence from resistant, transgenic plants. Molec Plant-Microbe Interact **13:** 125-128

McLean BG, Hempel FD, Zambryski PC (1997) Plant intercellular communication via plasmodesmata. Plant Cell **9:** 1043-1054

Medberry SL, Lockhart BE, Olszewski NE (1990) Properties of *Commelina yellow mottle virus*'s complete DNA sequence, genomic discontinuities and transcript suggest that it is a pararetrovirus. Nucleic Acids Res **18:** 5505–5513

Medberry SL, Lockhart BE, Olszewski NE (1992) The *Commelina yellow mottle virus* promoter is a strong promoter in vascular and reproductive tissues. Plant Cell **4:** 185-192

Medberry SL, Olszewski NE (1993) Identification of cis elements involved in C*ommelina yellow mottle virus* promoter activity. Plant J **3:** 619-626

Moreira A, Jones RAC, Fribourg CE (1980) Properties of a resistance-breaking strain of *Potato virus X*. Ann Appl Biol **95:** 93-103.

Morozov SY, Lukasheva LI, Chernov BK, Skryabin KG, Atabekov JG (1987) Nucleotide sequence of the open reading frames adjacent to the coat protein cistron in *Potato virus X* genome. FEBS Letters **213:** 438-442

Morozov SY, Miroshnichenko NA, Zelenina DA, Fedorkin ON, Solovijev AG, Lukasheva LI, Atabekov JC (1990) Expression of RNA transcripts of *Potato virus X* full-length and subgenomic cDNAs. Biochimie **72:** 677-684

Morozov SY, Solovyev AG (2003) Triple gene block: modular design of a multifunctional machine for plant virus movement. J Gen Virol **84:** 1351-1366

Nagano H, Mise K, Furusawa I, Okuno T (2001) Conversion in the requirement of coat protein in cell-to-cell movement mediated by the *Cucumber mosaic virus* movement protein. J. Virol. **75:** 8045-8053

Natilla A, Hammond RW, Nemchinov LG (2006) Epitope presentation system based on cucumber mosaic virus coat protein expressed from a *Potato virus X* -based vector. Arch Virol **151:** 1373-1386

Nelson RS, van Bel AJE (1998) The mystery of virus trafficking into, through, and out of vascular tissue. Prog Bot **59:** 476-533

Nurkiyanova KM, Ryabov EV, Kalinina NO, Fan Y, Andreev I, Fitzgerald AG, Palukaitis P, Taliansky M (2001) Umbravirus-encoded movement protein induces tubule formation on the surface of protoplasts and binds RNA incompletely and non-cooperatively. J Gen Virol **82:** 2579-2588

Oparka KJ, Roberts AG (2001) Plasmodesmata. A not so open-and-shut case. Plant Physiol **125:** 123-126

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

Oparka KJ, Santa Cruz S (2000) THE GREAT ESCAPE: Phloem transport and unloading of macromolecules. Annu Rev Plant Physiol Plant Mol Biol **51:** 323-347

Oparka KJ, Turgeon R (1999) Sieve elements and companion cells-traffic control centers of the phloem. Plant Cell **11:** 739-750

Parthasarathy MV, Larry HK (1976) Palm "wood". Wood Scie.Tech **10:** 247-271

Pascal E, Goodlove PE, Wu LC, Lazarowitz SG (1993) Transgenic tobacco plants expressing the geminivirus BL1 protein exhibit symptoms of viral disease. Plant Cell **5:** 795-807

Pascal E, Sanderfoot AA, Ward BM, Medville R, Turgeon R, Lazarowitz SG (1994) The geminivirus BR1 movement protein binds single-stranded DNA and localizes to the cell nucleus. Plant Cell **6:** 995-1006

Patrick JW (1997) PHLOEM UNLOADING: Sieve element unloading and post-sieve element transport. Annu Rev Plant Physiol Plant Mol Biol **48:** 191-222

Petty ITD, Jackson AO (1990) Mutational analysis of *Barley stripe mosaic virus* RNA [beta]. Virology **179:** 712-718

Pinck L (1975) The 5'-end groups of *Alfalfa mosaic virus* RNAs are M7G5'ppp5'Gp. FEBS Letters **59:** 24-28

Prokhnevsky AI, Peremyslov VV, Napuli AJ, Dolja VV (2002) Interaction between long-distance transport factor and Hsp70-related movement protein of *Beet Yellows Virus*. J Virol **76:** 11003-11011

Pruss G, Ge X, Shi XM, Carrington JC, Bowman Vance V (1997) Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. Plant Cell **9:** 859-868

Qin S, Ward BM, Lazarowitz SG (1998) The bipartite geminivirus coat protein aids BR1 function in viral movement by affecting the accumulation of viral single-stranded DNA. J Virol **72:** 9247-9256

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

Ryabov EV, Roberts IM, Palukaitis P, Taliansky M (1999) Host-specific cell-to-cell and long-distance movements of *Cucumber mosaic virus* are facilitated by the movement protein of *Groundnut rosette virus*. Virology **260:** 98-108

Sanderfoot AA, Ingham DJ, Lazarowitz SG (1996) A viral movement protein as a nuclear shuttle. The geminivirus BR1 movement protein contains domains essential for interaction with BL1 and nuclear localization. Plant Physiol **110:** 23-33

Sanderfoot AA, Lazarowitz SG (1995) Cooperation in viral movement: The Geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the cell periphery. Plant Cell **7:** 1185-1194

Santa Cruz S (1999) Perspective: phloem transport of viruses and macromolecules - what goes in must come out. Trends Microbiol **7:** 237-241

Santa Cruz S, Roberts AG, Prior DAM, Chapman S, Oparka KJ (1998) Cell-to-cell and phloem-mediated transport of *Potato Virus X*: The role of virions. Plant Cell **10:** 495-510

Schaad MC, Lellis AD, Carrington JC (1997) VPg of tobacco etch potyvirus is a host genotype-specific determinant for long-distance movement. J Virol **71:** 8624-8631

Scholthof HB, MorrisTJ., Jackson AO (1993) The capsid protein gene of *Tomato bushy stunt virus* is dispensable for systemic movement and can be replaced for localized expression of foreign genes. Molec Plant-Microbe Interact **6:** 309-322

Scholthof KB, Scholthof HB, Jackson AO (1995) The effect of defective interfering RNAs on the accumulation of *Tomato bushy stunt virus* proteins and implications for disease attenuation. Virology **211:** 324-328

Schultz ES (1944) The effect of latent mosaic (Virus X) on yield of potatoes. Am Potato J **21:** 278-283

Shi BJ, Palukaitis P, Symons RH (2002) Differential virulence by strains of *Cucumber mosaic virus* is mediated by the 2b gene. Mol Plant Microbe Interact **15:** 947-955

Sjolund RD (1997) The phloem sieve element: A river runs through it. Plant Cell **9:** 1137-1146

Skryabin KG, Kraev AS, Morozov SY, Rozanov MN, Chernov BK, Lukasheva LI, Atabekov JG (1988) The nucleotide sequence of *Potato virus X* RNA. Nucl. Acids Res. **16:** 10929-10930

Solovyev AG, Savenkov EI, Agranovsky AA, Morozov SY (1996) Comparisons of the genomic cis-elements and coding regions in RNA beta components of the hordeiviruses barley stripe mosaic virus, *Lychnis ringspot virus*, and *Poa semilatent virus*. Virology **219:** 9-18

Solovyev AG, Stroganova TA, Zamyatnin AA, Jr., Fedorkin ON, Schiemann J, Morozov SY (2000) Subcellular sorting of small membrane-associated triple gene block proteins: TGBp3-assisted targeting of TGBp2. Virology **269:** 113-127

Sonenberg N, Shatkin AJ, Ricciardi RP, Rubin M, Goodman RM (1978) Analysis of terminal structures of RNA from *Potato virus X*. Nucl. Acids Res. **5:** 2501-2512

Soto MJ, Chen L-F, Seo Y-S, Gilbertson RL (2005) Identification of regions of the Beet mild curly top virus (family Geminiviridae) capsid protein involved in systemic infection, virion formation and leafhopper transmission. Virology **341:** 257-270

Spillane C, Verchot J, Kavanagh TA, Baulcombe DC (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:** 76-84

Stadler R, Brandner J, Schulz A, Gahrtz M, Sauer N (1995) Phloem loading by the PmSUC2 sucrose carrier from plantago major occurs into companion cells. Plant Cell **7:** 1545-1554

Stols ALH, Hill-van der Meulen GW, Toen MKI (1970) Electron microscopy of Nicotiana glutinosa leaf cells infected with *Potato virus X*. Virology **40:** 168-170

Taiz L, Zeiger E (2002) Plant physiology, Ed 3 rd. Sinauer Associate, inc., Sunderland, MA

Tamai A, Kubota K, Nagano H, Yoshii M, Ishikawa M, Mise K, Meshi T (2003) Cucumovirus- and bromovirus-encoded movement functions potentiate cell-to-cell movement of tobamo- and potexviruses. Virology **315:** 56-67

Teri JM, David H, Plaisted RL (1977) The effect of *Potato virus X* on yield of the potato variety Hudson. Am Potato J **54:** 21-275.

Thompson MV, Holbrook NM (2003) Application of a single-solute non-steady-state phloem model to the study of long-distance assimilate transport. J Theor Biol **220:** 419-455

Turgeon R, Beebe DU (1991) The evidence for symplastic phloem loading. Plant Physiol **96:** 349-354

Turgeon R, Wimmers LE (1988) Different patterns of vein loading of exogenous [C]Sucrose in leaves of *Pisum sativum* and *Coleus blumei*. Plant Physiol **87:** 179-182

Van Bel AJE (1993) Strategies of phloem loading. Annu Rev Plant Physiol Plant Molec Biol **44:** 253-281

Van Bel AJE (2003) The phloem, a miracle of ingenuity. Plant Cell Environ **26:** 125-149

van Kesteren WJ, van der Schoot C, van Bel AJ (1988) Symplastic transfer of fluorescent dyes from mesophyll to sieve tube in stripped leaf tissue and partly isolated minor veins of *Commelina benghalensis*. Plant Physiol **88:** 667-670

Vance VB (1991) Replication of *Potato virus X* RNA is altered in coinfections with *Potato virus Y*. Virology **182:** 486-494

Vance VB, Berger PH, Carrington JC, Hunt AG, Ming Shi X (1995) 5' Proximal potyviral sequences mediate *Potato virus X* /potyviral synergistic disease in transgenic tobacco. Virology **206:** 583-590

Verchot-Lubicz J, Ye CM, Bamunusinghe D (2007) Molecular biology of Potexviruses: recent advances. J Gen Virol **88**: 1643 - 1655

Verchot-Lubicz J (2005) A new cell-to-cell transport model for Potexviruses. Mol Plant-Microbe Interact **18:** 283-290

Verchot J, Angell SM, Baulcombe DC (1998) In vivo translation of the triple gene block of *Potato virus X* requires two subgenomic mRNAs. J Virol **72:** 8316-8320

Voitsekhovskaja OV, Koroleva OA, Batashev DR, Knop C, Tomos AD, Gamalei YV, Heldt HW, Lohaus G (2006) Phloem loading in two Scrophulariaceae species. What can drive symplastic flow via plasmodesmata? Plant Physiol **140:** 383-395

Ward BM, Lazarowitz SG (1999) Nuclear export in plants. Use of geminivirus movement proteins for a cell-based export assay. Plant Cell **11:** 1267-1276

Wolf S, Lucas WJ, Deom CM, Beachy RN (1989) Movement protein of *Tobacco mosaic virus* modifies plasmodesmatal size exclusion limit. Science **246:** 377-379

Wong SM, Lee KC, Yu HH, Leong WF (1998) Phylogenetic analysis of triple gene block viruses based on the TGB 1 homolog gene indicates a convergent evolution. Virus Genes **16:** 295-302

Wooding FBP (1967) Endoplasmic reticulum aggregates of ordered structure. Planta **76:** 205-208

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

Zambryski P, Crawford K (2000) Plasmodesmata: Gatekeepers for cell-to-cell transport of developmental signals in plants. Annu Rev Cell Dev Biol **16:** 393-421

Zhu Y, Qi Y, Xun Y, Owens R, Ding B (2002) Movement of *Potato spindle tuber viroid* reveals regulatory points of phloem-mediated RNA traffic. Plant Physiol. **130:** 138-146

CHAPTER II

CLONING AND GENERATION OF TRANSGENIC PLANTS EXPRESSING *POTATO VIRUS X* (PVX) TGBp1, TGBp2 AND CP ORFs

ABSTRACT

PVX requires four proteins to facilitate virus cell-to-cell movement. These are named TGBp1, TGBp2, TGBp3, and CP. While the roles of these proteins in virus cell-to-cell movement have been established, their role in promoting virus vascular transport is less certain. Here we generated transgenic *Nicotiana tabacum* and *N. benthamiana* plants expressing the green fluorescent protein (GFP) coding sequence fused to TGBp1, TGBp2, and CP ORFs to study their role in PVX vascular transport. The fusions were separately introduced into the pCOI binary plasmid next to a companion cell specific promoter from *Commelina yellow mottle virus* (CoYMV) to study protein translocation through the phloem. Transgenic plants expressing GFP alone, GFP:GFP fusion, or transformed with pCOI vector alone were also prepared. Transgenic plants were produced via *Agrobacterium*-mediated transformation and were analyzed using PCR to verify the presence of the transgene. Microscopy was used to detect GFP expression. T1 plants were inoculated with PVX-GUS to determine if they were susceptible to virus infection.

INTRODUCTION

Plant viruses inoculated to epidermal cells move from the initially infected cell to adjacent epidermal and mesophyll cells through plasmodesmata. Viruses spread throughout the leaf and systemically through the plants by entering the phloem. Viruses enter the companion cells from surrounding parenchyma and move into the sieve elements via plasmodesmata. It is often suggested that viruses move long distance from inoculated leaves into young developing tissues following bulk flow translocation of photoassimilates (Ryabov et al., 1998; Ruiz-Medrano et al., 2001). Molecules involved in a wide range of physiological and developmental processes move through the phloem including proteins, carbohydrates, nucleic acids, defense signals, mRNA, small RNA, peptides, and phytohormones (Balachandran et al., 1997; Lucas and Wolf, 1999; Ruiz-Medrano et al., 1999, Ruiz-Medrano et al., 2001; Voitsekhovskaja et al., 2006). The path for viral entry and exit from the phloem is via plasmodesmata and this pathway is likely the same as the pathway for plant proteins and nucleic acids (Santa Cruz, 1999). New evidence suggests that proteins and nucleic acids are translocated by bulk flow as well as selectively which ensures reaching the target tissues (Aoki et al., 2005).

The path for entry of sucrose and carbohydrates into the sieve element is often apoplastic and requires active transporters in a mechanism described as phloem loading. The path for viruses, protein, and nucleic acid entry into the phloem from phloem parenchyma or companion cells is via plasmodesmata (Mezitt and Lucas, 1996; Lucas and Wolf, 1999; Oparka and Santa Cruz, 2000; Itaya et al., 2002) and may involve yet undescribed mechanisms that up or down regulates transfer of macromolecules into sieve elements (Gomez and Pallas, 2004). The numbers of plasmodesmata connections between the bundle sheath and parenchyma, between parenchyma and companion cells,

and then between companion cells and sieve elements are developmentally regulated, become fewer and fewer (Van Bel, 1993; Turgeon et al., 2001). The decline in plasmodesmata frequency from the bundle sheath toward the sieve element provides a mechanism to down regulate the flow of macromolecules into the sieve element (Van Bel, 1993; Wintermantel et al., 1997). However the plasmodesmal connections between companion cells and sieve elements have size exclusion limits (SELs) of at least 67 kDa (Stadler et al., 2005) which is larger than 1 kDa SEL of plasmodesmal connection between mesophyll cells and bundle sheath (Ding et al., 1992). This means that general diffusion of small molecules into sieve element is down regulated over distance while entry of large molecules synthesized primarily in the companion cells into the sieve element is greater.

Mutations in the TMV MP caused reduced systemic spread of the virus (Ding et al., 1996). The mutant TMV could move from cell-to-cell and from mesophyll cells into bundle sheath cells but fail to enter the phloem indicating that TMV may require one mechanism to move from cell-to-cell and another mechanism to spread from the bundle sheath into the phloem (Ding et al., 1996). Similarly when transgenic tobacco plants expressing truncated 2a replicase gene from *Cucumber mosaic virus* (CMV) were inoculated with CMV, systemic spread of virus was blocked at boundary between bundle sheath and vascular cells (Wintermantel et al., 1997). CMV RNA was synthesized at low levels indicating that optimal RNA amplification is necessary for virus entry into the phloem.

Nutrients move through the phloem in a source to sink direction by bulk flow translocation, meaning that they move from a site of high concentration to a site of low

concentration. Viruses, proteins, and nucleic acids are reported to follow nutrients along the same translocation stream (Oparka et al., 1999; Oparka and Santa Cruz, 2000). Initial studies conducted using the green fluorescent protein (GFP) expressed in companion cells of transgenic root stock showed fluorescence in sieve elements of wild type scion and GFP movement in sieve element followed the translocation stream to sink tissues (Imlau et al., 1999). Similarly, large number of phloem proteins such as PP1 (96 kDa), PP2 (48 kDa), CmmLe17 (17 kDa) from pumpkins were found to be carried passively by bulk (Golecki et al., 1999; Aoki et al., 2005). Viruses, proteins, and nucleic acids may also rely on a destination selective mechanism of phloem translocation (Itaya et al., 2002; Aoki et al., 2005). The destination selective mechanism suggests that certain macromolecules are targeted to specific locations by chaperones present in the phloem. The CmPP16-1 (16. 5 kDa) and CmPP16-2 (15. 6 kDa) proteins which were purified from phloem exudates (Xoconostle et al., 1999) were translocated to shoots passively by bulk flow; however, root ward translocation of these proteins was selectively controlled (Aoki et al., 2005). The root ward translocation of CmPP16-1 was up regulated when a complex with phloem sap proteins were formed suggesting that CmPP16-1 chaperones nucleic acids through the phloem and accumulate to high levels in root. However, CmPP16-2 did not accumulate in the root, suggesting that proteins are selectively degraded in locations where they are not targeted or needed. Destination selective translocation proteins and RNA might contribute to regulate developmental and defense responses at distant locations (Aoki et al., 2005).

In sink leaves, viruses and proteins exit mainly through minor veins via plasmodesmata following other macromolecules (Carrington et al., 1996; Baluska et al.,

2001). In tobacco and Arabidopsis, minor veins lack bundle sheath cells and virus is often seen to escape directly from the phloem parenchyma into mesophyll cells. The plasmodesmal SEL in sink tissues is larger than source tissue creating a greater opportunity for proteins and protein complexes to diffuse out of the phloem into neighboring tissues (Oparka et al., 1999; Asuka et al., 2000). For example, transgenic Arabidopsis plants expressing GFP (27 kDa in size) from the phloem specific AtSUC2 promoter showed fluorescence can be detected in mesophyll cells of sink leaves (Imlau et al., 1999). Viruses, proteins, and nucleic acids exit the phloem in sink leaves or other targeted locations (Oparka, 1990; Zhang et al., 2006). For example in a study to describe phloem unloading of proteins and nucleic acid using either the phloem-mobile carboxyfluorescein (CF) dye or a recombinant PVX virus containing GFP (Roberts et al., 1997), fluorescence due either to CF dye or PVX-GFP spread from the phloem of minor veins into mesophyll cells in sink leaves. Similarly, PSTVd which is entirely dependent on host factors for movement, showed the viroid could not exit the phloem into sink tissues (Zhu et al., 2002). These results suggest that viruses, proteins, and nucleic acids exit from sieve element into sink tissues in a manner that is regulated.

While the roles of the PVX triple gene block proteins in virus cell-to-cell movement have been studied, there are few studies exploring their role in virus vascular transport (Angell and Baulcombe, 1995; Santa Cruz et al., 1998; Krishnamurthy et al., 2002; Krishnamurthy et al., 2003; Mitra et al., 2003; Howard et al., 2004; Ju et al., 2005; Verchot et al, 2007). Evidence that PVX TGBp1 increases plasmodesmal SEL (Santa Cruz et al., 1998; Howard et al., 2004) independent of TGBp2, TGBp3 or CP, and binds viral nucleic acids suggests that it plays a role in transport of viral nucleic acids between

cells and possibly through the vasculature. PVX TGBp2 and TGBp3 are membrane binding proteins (Tamai and Meshi, 2001; Krishnamurthy et al., 2003; Ju et al., 2005; Ju et al., 2007), suggesting that cell to cell and vascular movement of PVX might involve membrane-mediated processes. Since mutations in any one of the PVX triple gene block or CP ORFs is sufficient to block virus vascular transport, it is likely that these proteins interact to promote virus entry into, translocation through, and exit from the phloem (Santa Cruz et al., 1998; Yang et al., 2000). To study phloem translocation of potexviruses, healthy scions were grafted onto rootstocks expressing triple gene block or CP coding sequences (Santa Cruz et al., 1998; Lough et al., 2001). Grafted plants were inoculated with mutant viruses and tested for their ability to complement viral long distance movement defects. A model was proposed in which TGBp1 and CP bind viral RNA forming a ribonucleoprotein complex which traffics through the phloem to sink tissues (Lough et al., 2000; Lough et al., 2001).

To study activities of PVX TGBp1, TGBp2, and CP within the phloem, transgenic plants were prepared using the companion cell specific CoYMV promoter, to express GFP fusions (Medberry et al., 1992; Medberry and Olszewski, 1993; Matsuda et al., 2002; Gittins et al., 2003). GFP fluorescence was used to visualize protein translocation through and exit from the phloem.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

All plasmids were constructed using *Escherichia coli* strain JM109 (Sambrook et al., 1989). The QIAGEN RNA/DNA Midi Kit (Qiagen, Inc., Valencia, CA) and the Wizard Plus Miniprep DNA Purification System (Promega Corp, Madison, WI) were used to purify plasmids from *E. coli.* The pCOI binary plasmid contains the CoYMV promoter, *Bam*HI and *Sma*I restriction sites, and a Nos terminator (Fig. 1A). The pCOI and pCOI-GFP:GFP plasmids were obtained from Dr. Biao Ding (Ohio State University) (Itaya et al. , 2002). The pCOI-GFP plasmid was prepared by Dr. Chang-Ming Ye in our laboratory. The pRTL2-GFP, -GFP:TGBp1, -GFP:TGBp2, and -GFP:CP plasmids contain the GFP gene fused to the PVX TGBp1, TGBp2 and coat protein (CP) genes respectively, and were described previously (Krishnamurthy et al., 2002; Krishnamurthy et al., 2003). Each pRTL2 plasmid was digested with *Bam*HI and *Eco*RI restriction enzymes. The digested fragments were made blunt using T4 DNA polymerase according to manufacturer's instructions (Promega Corp.). The pCOI plasmid was digested with *Sma*I and then treated with Calf Intestinal Alkaline Phosphatase (CIAP; Promega Corp.) to dephosphorylate the 5'ends and prevent recircularization of the plasmid. GFP:TGBp1 (~1.5 kb); GFP:TGBp2 (~1 kb) and GFP:CP (~1.5 kb) fragments, and CIAP-treated pCOI vector were gel purified using the Nucleospin Extraction Kit (BD Biosciences Clontech, Palo Alto, CA) and then ligated by incubating at 14^0C overnight with T4 DNA ligase (Promega Corp.). *E. coli* was transformed by electroporation using 2 mm cuvettes and the Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Voltage, resistance, and capacitance were adjusted to 2. 5 kV, 200 Ω, 25 μFD, respectively. The pCOI-

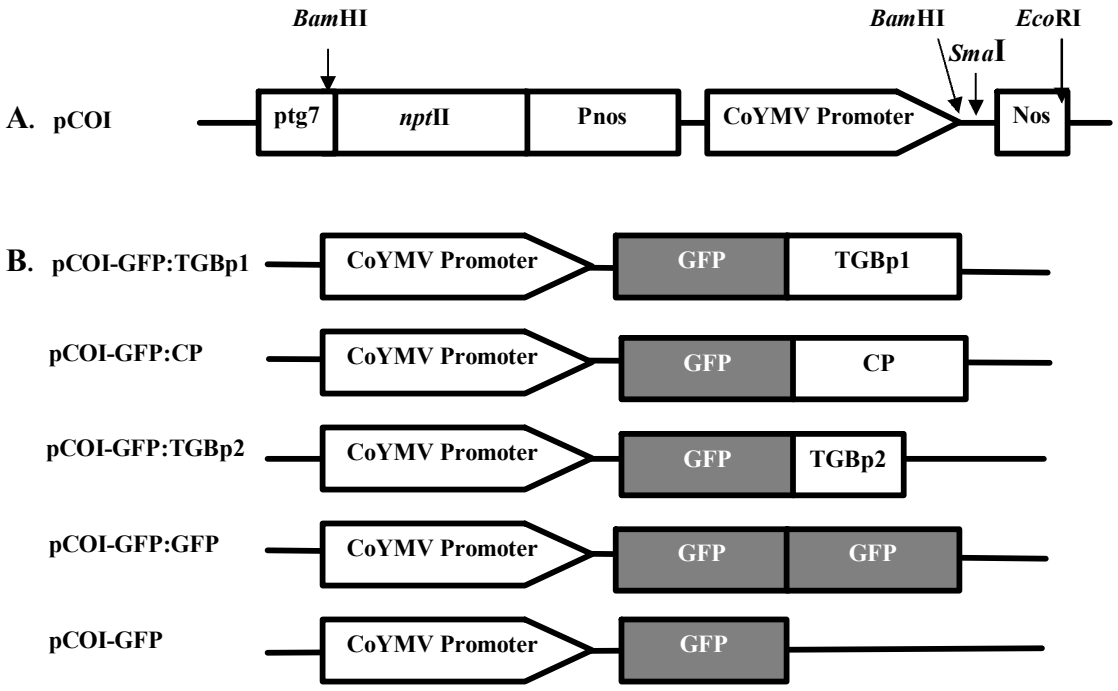


Figure 1. Schematic representations of pCOI plasmids used in this study. White arrows in each panel represent the 1 kb CoYMV promoter. A, The pCOI plasmid contains the *neomycin phosphotransferase II* (*nptII*) gene adjacent to the nopaline synthase promoter (Pnos) and the ptg7 terminator. Between the CoYMV promoter and nos terminator are *Bam*HI and *Sma*I restriction sites used for insertion of foreign genes. B, Diagrammatic representation of pCO1-GFP:TGBp1, -GFP:TGBp2, -GFP:CP, -GFP:GFP, and GFP plasmids. The white boxes represent PVX coding sequences and the gray boxes represent GFP coding sequences.

GFP:TGBp1, GFP:TGBp2, -GFP:CP plasmids (Fig. 1B) were verified by colony polymerase chain reactions (PCR), restriction analysis, and DNA sequencing.

Transformation of *Agrobacterium tumefaciens* Strain LBA4404

Each pCOI plasmid was introduced into *A. tumefaciens* strain LBA4404 by electroporation using 2 mm cuvettes and the Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Voltage, resistance, and capacitance were adjusted to 2.5 kV, 200 Ω 25 μFD, respectively. Following electroporation, *A. tumefaciens* strain LBA4404 was grown on YEP medium (yeast extract, 10 g L^{-1}; peptone, 10 g L^{-1}; NaCl, 5 g L^{-1}; 15 g L^{-1} agar; pH 7. 0) containing streptomycin (50 μg mL^{-1}) and kanamycin (100 μg mL^{-1}). Colony PCR was used to verify *A. tumefaciens* strain LBA4404 that contained the desired plasmids.

Plant Growth and Transformation

N. tabacum cv. Petit Havana seeds were surface sterilized with 10 % Clorox bleach for 10 minute, washed twice with sterile ddH_2O and then transferred to germination medium (4.6 g L^{-1} Murashige and Skoog salts (Sigma-Aldrich), 30 g L^{-1} sucrose, 0.59 g L^{-1} MES, 1 g L^{-1} B5 vitamin, and 6 g L^{-1} Phytoagar). Seeds were grown under a long-day cycle (16/ 8 h day/ night) and 23 ^{o}C under 40 W cool white fluorescent light bulbs. After five weeks, seedlings were transferred into separate magenta boxes containing germination medium.

N. tabacum leaf discs were excised and transformed using *Agrobacterium tumefaciens* (Horsch et al., 1985) containing pCOI-GFP:TGBp1, pCOI-GFP:TGBp2, pCOI-GFP:CP,

pCOI-GFP:GFP, pCOI-GFP or pCOI plasmids. Leaves were taken from 68 days old plants, cut into segments and combined with 10 ml culture of Agrobacterium for 10 min. Leaf segments were blotted on sterile filter paper, transferred to plates containing germination medium, and incubated in the dark for 2 days at room temperature. Leaf segments were washed three times with MS liquid medium (4.6 g L^{-1} Murashige and Skoog salts (Sigma-Aldrich) and 30 g L^{-1} sucrose) and transferred to plates containing *N. tabacum* shooting medium (4.6 g L^{-1} Murashige and Skoog salts (Sigma-Aldrich), 30 g L^{-1} sucrose, 0.59 g L^{-1} MES, 1 g L^{-1} B5 vitamin, 6 g L^{-1} Phytoagar, 0.1 mg L^{-1} α-naphthalene acetic acid (NAA), 1mg L^{-1} 6-benzyl amine purine (BAP), 250 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin). After two weeks the cultures were transferred to fresh *N. tabacum* shooting medium. After approximately 4 weeks, shoots were transferred to *N. tabacum* rooting medium (2.3 g L^{-1} Murashige and Skoog salts (Sigma-Aldrich), 5 g L^{-1} sucrose, 6 g L^{-1} Phytoagar, 200 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin).

Transformation of *N. benthamiana* was conducted using leaves of two-week-old grown plants grown in the green house. Young leaves were collected; surface sterilized with 10 % Clorox bleach for 10 min and washed three times with sterile ddH_2O. *Agrobacterium* and leaf segments were co-cultivated on plates containing *N. benthamiana* shooting medium (4.3 g L^{-1} Murashige and Skoog salts (Sigma-Aldrich), 30 g L^{-1} sucrose, 1 g L^{-1} B5 vitamin, 8 g L^{-1} agar, 0.5mg/l BAP) for 3 days in the dark. Leaf segments were washed three times with MS liquid medium and transferred to plates containing *N. benthamiana* shooting medium (4.3 g L^{-1} Murashige and Skoog salts (Sigma-Aldrich), 30 g L^{-1} sucrose, 1 g L^{-1} B5 vitamin, 8 g L^{-1} agar, 0.18 mg L^{-1} NAA, 1.8

mg L^{-1} BAP, 250 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin) for two weeks. Then leaf disks were transferred to fresh *N. benthamiana* shooting medium for approximately 4 weeks. Shoots were transferred to *N. benthamiana* rooting medium (4.3 g L^{-1} Murashige and Skoog salts (Sigma-Aldrich), 5 g L^{-1} sucrose, 6 g L^{-1} Phytoagar, 250 mg L^{-1} carbenicillin, and 100 mg L^{-1} kanamycin).

Transgenic *N. tabacum* and *N. benthamiana* plantlets were transferred from rooting medium to magenta boxes containing sterile soil and then transferred to pots in the greenhouse.

Analysis of GFP Expression in Transgenic Plants

Leaves of T0 plants were observed under the microscope when the plants were first transferred to soil. A Nikon E600 epifluorescence microscope equipped with a B2A filter (Nikon Instrument Inc, Tokyo, JP) was used to verify GFP expression. Table I shows the number of T0 plants that were positive for GFP expression. T1 plants were grown from each line (Table I) and then cross sections of petioles and stems were examined microscopically for GFP expression. Most T0 plants that showed GFP expression in the vasculature were then analyzed by PCR to verify presence of the transgene.

PCR Analysis of Ttransgenic Plants

DNeasy Plant Mini Kit (QIAGEN Inc.) was used to isolate DNA from T0 and T1 transgenic plants. To PCR amplify the full length of GFP:TGBp1, GFP:TGBp2 ,

GFP:CP, GFP:GFP and GFP inserts we used a forward primer that overlapped with the 5' end of the GFP coding sequence (GCG CCG CCC GGG ATG GTG AGC AAG GGC GAG GAG CTG). The reverse primers overlapped the 3' ends of the PVX TGBp1 (CTA TGG CCC TGC GCG GAC ATA TGT CAA TCC CTT TG), PVX TGBp2 (GCG CCG GGT ACC CTA ATG ACT GCT ATG ATT GTT ACC), PVX CP (CTC GAG TGA CAG CTG CAT CTA GGC TGG CAA AG), or GFP (GCG CCG GGT ACC TTA CTT GTA CAG CTC GTC CAT GCC) coding sequences. PCR reactions were carried out at 95^{o}C for 2 min, 95^{o}C for 45 s, 60^{o}C for 30 s, 72^{o}C for 1 to 1.5 min, for 35 cycles and 10 mL of the reaction were run in 0.9% agarose gel.

To verify the presence of the CoYMV promoter, we employed PCR using a forward primer overlapping the 5' end (CGG TAT GCC GGT TCC CAA GCT TTA TT) and a reverse primer overlapping the 3' end (GCG CCG CTC GAG CTT GTT GTG TTG GTT TTC TAA GCT) of the CoYMV promoter. PCR reactions were carried as described above.

In vitro Transcription and Plant Inoculations

Nontransgenic or T1 transgenic *N. tabacum* and *N. benthamiana* were inoculated with infectious PVX-GUS transcripts or infected plant sap. The mMessage mMachine T7 kit (Ambion Inc., Austin, TX) was used to prepare infectious PVX-GUS transcripts. One μg *Spe*I linearized plasmids was added to each transcription reaction. Transcription reactions were directly used to inoculate plants in the greenhouse. Leaves were dusted with carborundum and then rub-inoculated with 2-5 μL (between 30-150 μg) of the

transcripts (Chapman et al., 1992; Baulcombe et al., 1995). Following inoculation, plants were misted with water.

For some experiments, PVX-GUS infected *N. benthamiana* leaves were excised and ground in a mortar and pestle with 2 vol (w/v) 0.1 M phosphate buffer, pH 8.0 (94 mL of 1 M K_2HPO_4, 6 mL of 1M KH_2PO_4, plus 900 mL ddH_2O) plus 0.2% β-mercaptoethanol (v/v) and 10% ethanol (v/v) and used for rub inoculation as described above for the transcripts.

GUS Assay

To determine the vascular spread of PVX-GUS, leaf samples were collected from inoculated plants and vacuum infiltrated with a solution containing 0.5 g L^{-1} X-Gluc (dissolved in 10 vol (w/v) DMF), 0.1 M sodium phosphate buffer pH 7.0 (57.7 mL of 1M Na_2HPO_4, 42.3 mL of 1M NAH_2PO_4, plus 900 mL ddH_2O), 0.19 g L^{-1} EDTA, 0.232 g L^{-1} $K_4Fe(CN)_6$ -$3H_2O$ and 0.164 g L^{-1} $K_3Fe(CN)_6$, and 1% Triton X-100. Leaves were then transferred to an Equatherm incubator (Curtin Matheson Scientific Inc., Houston TX) and maintained overnight at 37 ^oC. Leaves showing a blue precipitate due to GUS activity were visually counted and scanned using Hewlett Packard Scanjet 4570c (Hewlett-Packed Company, L.P., Palo Alto, CA). Images were compiled using Adobe Photoshop CS software (Adobe Systems, Inc., San Jose, CA).

DAS-ELISA

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to verify PVX infection in upper, non-inoculated leaves. The PathoScreen kit

(Agdia Inc., Elkhart, Indiana) specifically designed for detecting PVX was used. Leaf tissues (0.1 g) and 5 mL Agdia extraction buffer were placed in an extraction bag and samples were homogenized with a HOMEX 6 homogenizer which can process five bags simultaneously (Bioreba Ag, CH-4153 Reinach BL1). Aliquots (100 μL) of each sample then were transferred to the microtitre plates provided with the PathoScreen kit. Samples were developed for 1 h using PNP tablets which react with alkaline phosphatase to produce a yellow precipitate. Plates were visually examined and scanned using a HP Scanjet 4570c scanner (Hewlett-Packed Company, LP). Images were compiled using Adobe Photoshop CS software (Adobe Systems, Inc.)

RESULTS

Transgenic Plants Development

Transgenic *N. tabacum* and *N. benthamiana* expressing GFP fused to the 5' ends of PVX TGBp1, TGBp2 or CP under the control of a phloem specific promoter were made. The fused genes were inserted into the pCOI binary plasmid next to the companion cell specific CoYMV promoter (Fig. 1A). Transgenic plants expressing GFP:GFP, GFP or containing the pCOI vector alone were prepared. GFP was used as a visual marker to verify transgene expression and to study subcellular accumulation of the fusion proteins (Fig. 1B).

Transformation experiments were conducted with the goal to generate at least three transgenic plants of each *Nicotiana* species with each construct expressing high levels of GFP. Transformation experiments were carried out in three phases for two reasons: 1) all the clones were not made at the same time and 2) handling of all transgenic plants at the same time was challenging. During the first phase transgenic *N. tabacum* (*Nt*) lines were prepared: *Nt*GFP:TGBp1, *Nt*GFP:CP and *Nt*GFP:GFP. During the second phase *N. tabacum* and *N. benthamiana* (*Nb*) *Nt*GFP:TGBp2 *Nb*GFP:TGBp1 and *Nb*GFP:CP were prepared and in the third phase *Nt*GFP, *Nt*COI , *Nb*GFP:TGBp2, *Nb*GFP and *Nb*COI were prepared (Table 1).

To determine the transformation efficiency of *N. tabacum* and *N. benthamiana,* leaf discs from T0 plants that were regenerated on kanamycin containing medium were analyzed by PCR to detect the transgene and leaves were excised and examined microscopically to detect GFP expression in the veins. Table I shows the total number of

plants regenerated and the number of plants containing the transgene as determined by PCR and epifluorescence microscopy.

PCR analysis of *N. tabacum* plants showed 44% of *Nt*GFP:TGBp1 regenerated plants (11/25) contained the appropriate transgene. One hundred percent of *Nt*GFP:TGBp2 (5/5), 62% of *Nt*GFP:CP (20/32), 87.5% *Nt*GFP:GFP (14/16), and 100% *Nt*COI (10/10) were positive for each appropriate transgene by PCR (Table 1, Fig. 2). Among *N. benthamiana* transformants regenerated on kanamycin containing medium, 100% of *Nb*GFP:TGBp1 (6/6), *Nb*GFP:TGBp2 (5/5), *Nb*GFP:GFP (8/8) and *Nb*COI (5/5) were shown by PCR to contain the appropriate transgene. Eighty % of *Nb*GFP:CP(4/5) transformants were PCR positive for the transgene (Table 1; Fig. 2).

Plants that were found to be positive by PCR were also examined using epifluorescence microscopy to detect GFP expression (Table 1, Fig 3). Between 40% and 100% of examined plants were positive for GFP expression in the veins of young leaves (Table 1). All T0 transgenic lines showing GFP fluorescence and tested positive by PCR for the transgene were grown to collect T1 seeds for further experimentation. All T0 *Nt*COI and *Nb*COI lines which were positive by PCR for the presence of the CoYMV promoter were also grown to collect T1 seeds. Each transformant using a particular construct was serially designated as a suffix next to transgene. For example all 5 *Nt*GFP:TGBp1 were named as *Nt*GFP:TGBp1-1, *Nt*GFP:TGBp1-2, *Nt*GFP:TGBp1-3, *Nt*GFP:TGBp1-4 and *Nt*GFP:TGBp1-5.

Based on intensity of GFP expression two T1 lines from *Nt*GFP:TGBp1 (*Nt*GFP:TGBp1-101 & *Nt*GFP:TGBp1-113); *Nt*GFP:TGBp2 (*Nt*GFP:TGBp2-102 & *Nt*GFP:TGBp2-105), *Nt*GFP:CP (*Nt*GFP:CP-117 & *Nt*GFP:CP-149), *Nt*GFP:GFP

(*Nt*GFP:GFP-105 & *Nt*GFP:GFP-106), *Nb*GFP:TGBp1 (*Nb*GFP:TGBp1-201 & *Nb*GFP:TGBp1-203), *Nb*GFP:TGBp2 (*Nb*GFP:TGBp2-204 & *Nb*GFP:TGBp2-208), *Nb*GFP:CP (*Nb*GFP:CP-202 & *Nb*GFP:CP-207), *Nb*GFP:GFP (*Nb*GFP:GFP-201 & *Nb*GFP:GFP-204) were selected and five plants from each line, i.e., 10 plants per transgene were tested for GFP expression and 80, 90, 85, 95, 85, 90, 85 and 90 %, respectively of *Nt*GFP:TGBp1, *Nt*GFP:TGBp2, *Nt*GFP:CP, *Nt*GFP:GFP, *Nb*GFP:TGBp1, *Nb*GFP:TGBp2, *Nb*GFP:CP and *Nb*GFP:GFP plants were expressed GFP (data not shown). All of the above T1 lines then were further advanced to the T2 generation and seed were collected for studying protein translocation and exit from the phloem in the next chapter.

Table I. *Transformation and analysis of tobacco plants transformed with six plasmids.*

Transgenic *N. tabacum* and *N. benthamiana* plants express GFP or GFP containing fusions in CCs**.** Control plants were transformed with pCOI only. Plants were tested by PCR to verify the presence of the transgene and promoter. Plants were screened microscopically to verify GFP expression in the vasculature in leaves, petioles, and stems.

	Total No.	No. T0 plants positive	
Plant [a]	T0 plants	PCR[b]	GFP[c]
*Nt*GFP:TGBp1	25	11	11
*Nt*GFP:TGBp2	5	5	3
*Nt*GFP:CP	32	20	9
*Nt*GFP:GFP	16	14	6
*Nt*GFP	17	ND[d]	10
*Nt*COI	10	10	0
*Nb*GFP:TGBp1	6	6	4
*Nb*GFP:TGBp2	5	5	5
*Nb*GFP:CP	5	4	4
*Nb*GFP:GFP	8	8	5
*Nb*GFP	14	ND	12
*Nb*COI	7	5	0

[a]All transgenic lines labeled with Nt are *N. tabacum* and all transgenic lines labeled with Nb are *N. benthamiana*.
[b]. Number of T0 plants which were shown to contain the CoYMV promoter and transgene using PCR.
[c] Number of plants that shown GFP expression in the veins when analyzed using an epifluorescence microscope.
[d] ND= not determined**.** For these transgenic lines PCR was used to verify transgene in T1 plants

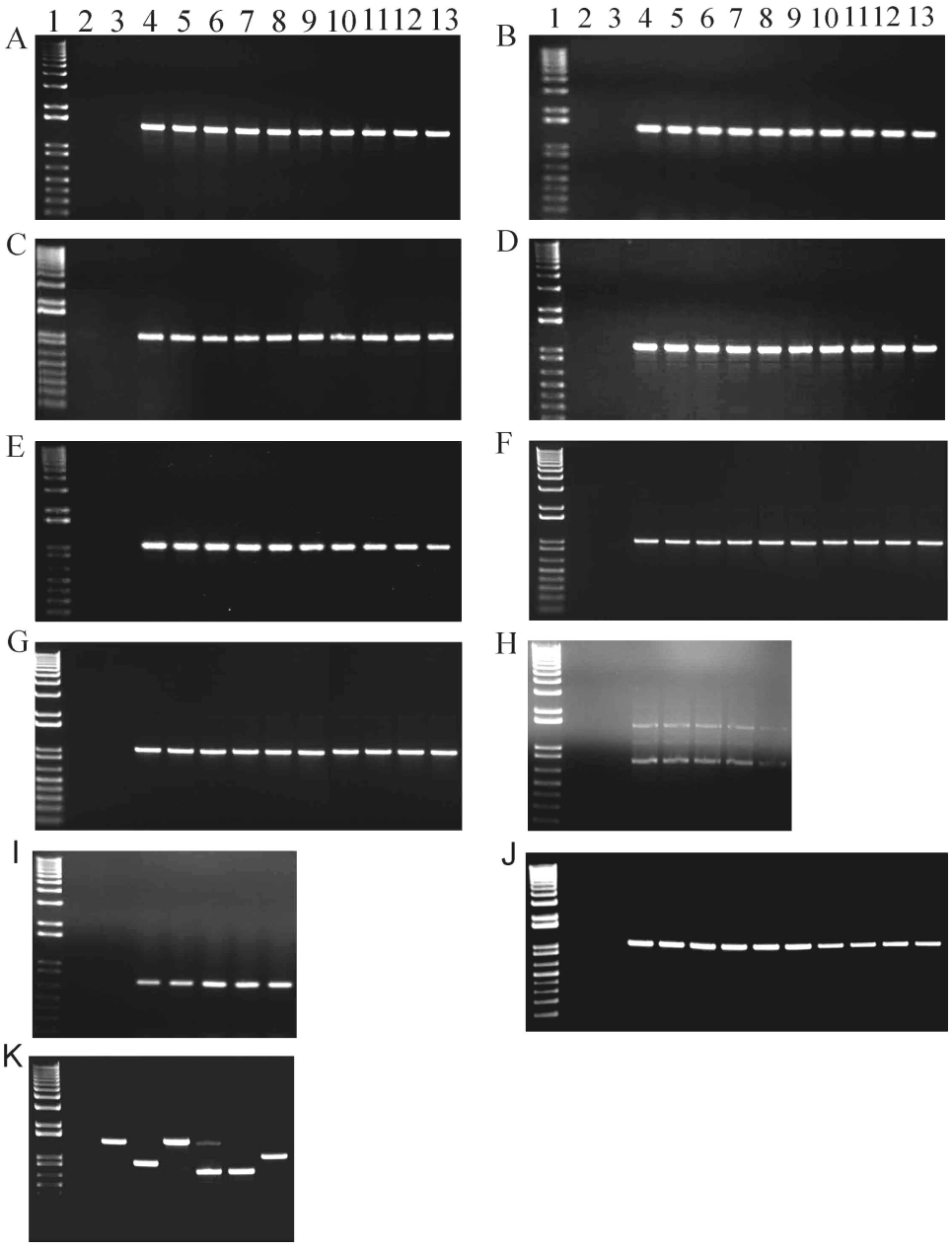


Figure 2. PCR analysis of genomic DNA from transgenic *N. tabacum* (A), (C), (E), (G) and *N. benthamiana* (B), (D), (F), (H). In all panels, lane 1 contains 1 Kb Plus DNA ladder (Invitrogen, Carlsbad, California), lane 2 (except panel I) contains PCR products derived from nontransgenic *N. tabacum* or *N. benthamiana* plants, lane 3 (except panel I) contain the products of PCR reactions run without a DNA template. A, B, PCR products

from five *Nt*GFP:TGBp1 and *Nb*GFP:TGBp1 (lanes 4-8), five *Nt*GFP:CP and *Nb*GFP:CP (lanes 9-13) T0 plants corresponding to the GFP:TGBp1 and GFP:CP fusions (both 1500 bp). C, D, five *Nt*GFP:TGBp1 and *Nb*GFP:TGBp1 (lanes 4-8), five *Nt*GFP:CP and *Nb*GFP:CP (lanes 9-13) T0 plants corresponding to the CoYMV promoter (1100 bp). E, five *Nt*GFP:TGBp2 (lane 4-8) and five *Nb*GFP:TGBp2 (lane 9-13) T1 transgenic plants corresponding to the GFP:TGBp2 fusion (1020 bp). F, G, five *Nt*GFP:TGBp2 and *Nt*GFP:GFP (lane 4-8) and five *Nb*GFP:TGBp2 and *Nb*GFP:GFP (lane 9-13) T1 transgenic plants corresponding to the CoYMV promoter (1100 bp). H, five *Nb*GFP:GFP T1 transgenic plants (lane 4-8) corresponding to the GFP:GFP fusion (1500 and 750 bp). I, PCR product without a DNA template (lane 2), and from five *Nb*GFP T1 transgenic plants (lane 4-8) corresponding to GFP coding sequence (750 bp). J, five *Nt*COI (lane 4-8) and five *Nb*COI (lane 9-13) T1 transgenic plants corresponding to the CoYMV promoter (1100 bp). K, PCR products derived from pCOI plasmids: lane 2 buffer; lane 3-8, -GFP:TGBp1 (1500 bp), -GFP:TGBp2 (1000 bp), -GFP:CP (1500), -GFP:GFP (1500 bp and 750 bp); -GFP (750 bp), and -CoYMV promoter (1100 bp).

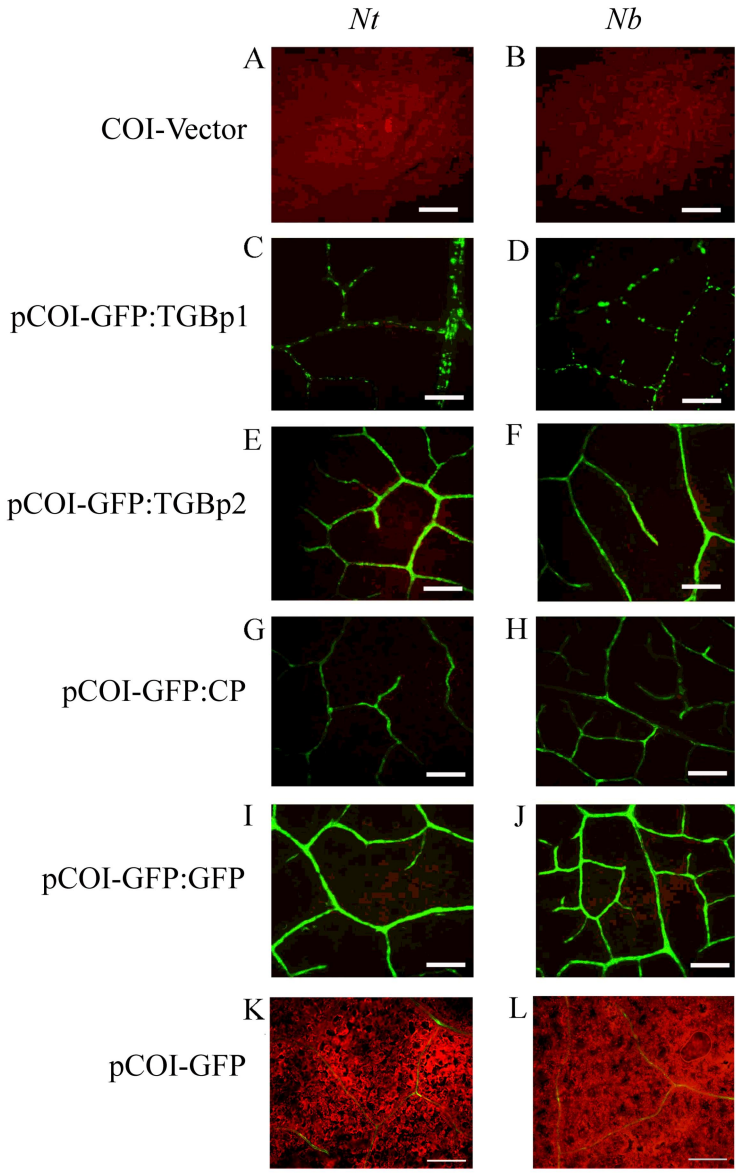


Figure 3. Images of transgenic tobacco leaves expressing GFP in the vasculature of T0 plants. Images in (A), (C), (E), (G), (I), and (K) are from transgenic *N. tabacum* and in (B), (D), (F), (H), (J), and (L) are from *N. benthamiana*. The identity of each transforming plasmid is indicated on the left. Bars represent 200µm.

Transgenic Plants Inoculation

To determine if the transgenic plants are susceptible to PVX infection, 15 plants expressing GFP were selected inoculated with PVX-GUS. Out of 15 plants five plants from T1 lines of *Nt*GFP:TGBp1, *Nt*GFP:TGBp2, *Nt*GFP:CP, *Nb*GFP:TGBp1 *Nb*GFP:TGBp2 and *Nb*GFP:CP were inoculated with infectious PVX-GUS transcripts, and infection was verified by symptom and GUS assay. All other plants were inoculated by plant sap taken from PVX-GUS inoculated tobacco plants and infection was verified by PCR and DAS-ELISA. Non transgenic *N. tabacum* and *N. benthamiana* plants were also inoculated as controls (Table II, Figs 4 and 5).

Assays for symptom development (Table II), GUS activity (Table II, Fig 4) and DAS-ELISA (Fig. 5) in non inoculated leaves of transgenic and non transgenic plants revealed that the transgenic lines are susceptible for PVX. The transgenes never blocked the spread of the virus in both transgenic and non transgenic *N. tabacum* and *N. benthamiana*.

Table II. *Transgenic and nontransgenic tobacco plants were inoculated with PVX-GUS to determine susceptibility.*

Plants were monitored for symptom expression for 15 days. Symptoms appeared on nontransgenic and transgenic *N. tabacum* between 8 and 15 days post inoculation. Symptoms appeared on nontransgenic and transgenic *N. benthamiana* between 4 and 6 days post inoculation. Systemic spread was verified using histochemical assay for GUS expression or DAS-ELISA to detect PVX CP. Numbers in each column represents the total number of plants assayed for GUS activity and DAS-ELISA and tested positive

	Total	No. of inoculated plants	
	No. of plants	GUS[a]	ELISA[b]
Tobacco plants	Infected	+(ve)	+(ve)
*Nt*GFP:TGBp1-101	15(15)	5(5)	10(10)
*Nt*GFP:TGBp2-102	15(15)	5(5)	10(10)
*Nt*GFP:CP-117	14(15)	4(5)	10(10)
*Nt*GFP:GFP-105	15(15)	5(5)	10(10)
*Nt*GFP-108	15(15)	ND[c]	15(15)
*Nt*COI-107	15(15)	ND	15(15)
N. tabacum	15(15)	5(5)	10(10)
N. tabacum (Mock)	15(15)	0(5)	0(10)
*Nb*GFP:TGBp1-201	15(15)	5(5)	10(10)
*Nb*GFP:TGBp2-204	15(15)	5(5)	10(10)
*Nb*GFP:CP-207	15(15)	5(5)	10(10)
*Nb*GFP:GFP-201	15(15)	5(5)	10(10)
*Nb*GFP-205	10(10)	ND	10(10)
*Nb*COI-203	10(10)	ND	10(10)
N. benthamiana	15(15)	5(5)	10(10)
N. benthamiana (Mock)	15(15)	0(5)	5(10)

[a] Plants inoculated with PVX-GUS transcript were tested for disease spread using GUS assay.
[b] Plants inoculated with virus extract were tested for disease spread using DAS-ELISA.
[c]ND =Not determined**.** For these transgenic lines ELISA was used to verify infection. Numbers in parenthesis are the total number of plants tested

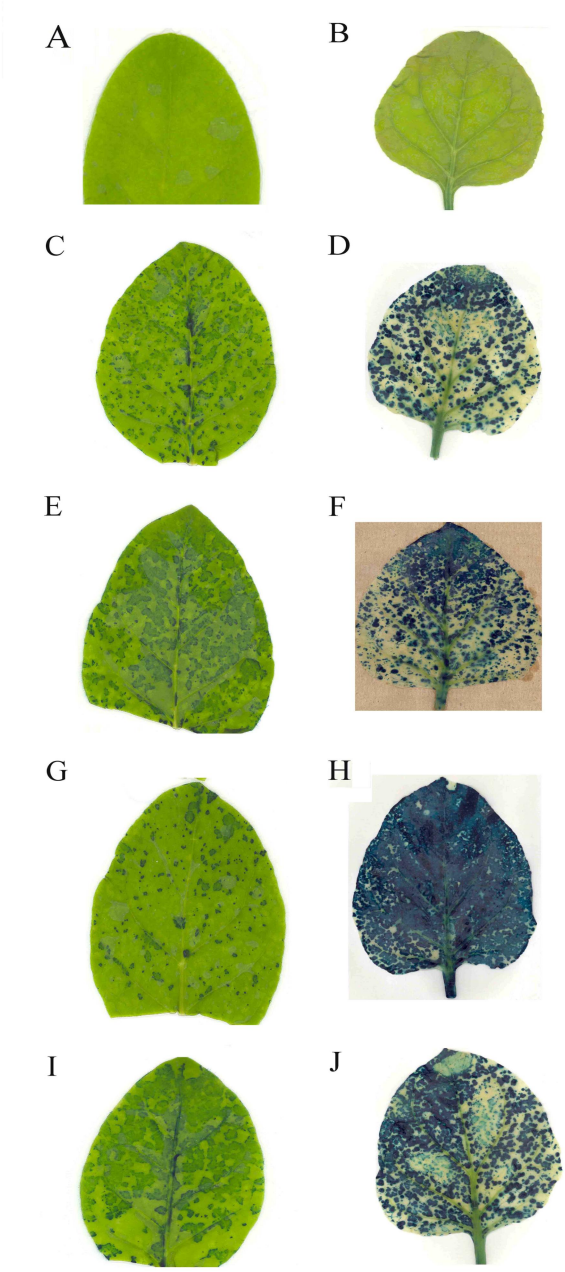


Figure 4. Histochemical assay for GUS expression in systemic leaves of transgenic plants infected with PVX-GUS. Each picture represents non inoculated upper leaves of non-infected *N. tabacum* (A), non-infected *N. benthamiana* (B), infected *N. tabacum* (C), infected *N. benthamiana* (D), *Nt*GFP:TGBp1-101 (E), *Nb*GF:TGBp1-201 (F), *Nt*GFP:TGBp2-102 (G), *Nb*GFP:TGBp2-204 (H), *Nt*GFP:CP-117 (I) and *Nb*GFP:CP-207 (J).

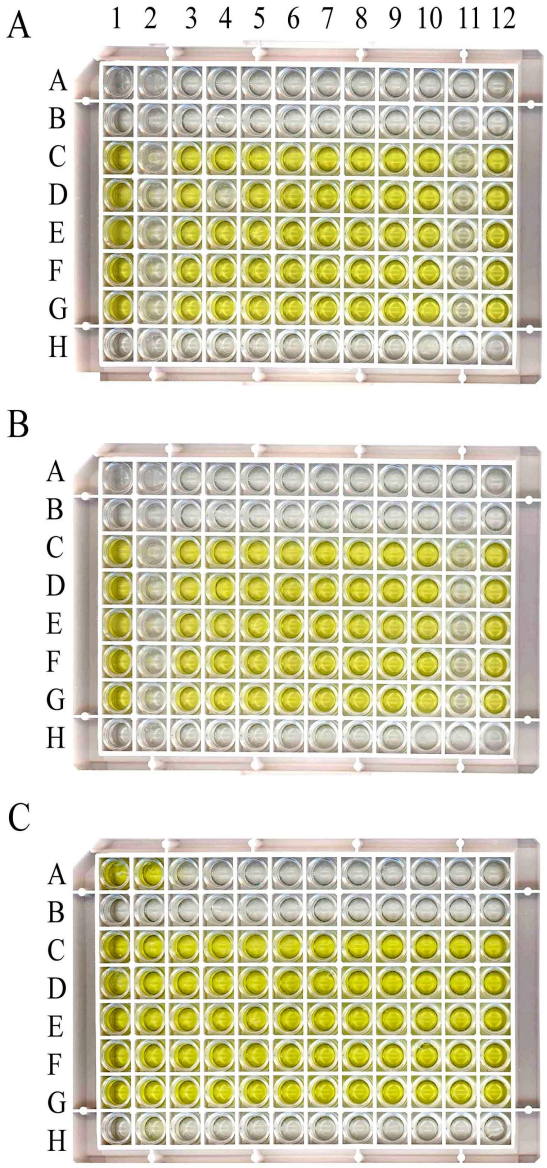


Figure 5. DAS-ELISA using leaf extracts from transgenic plants inoculated with virus extract and non transgenic controls. Plants inoculated with virus extract were tested for disease spread using DAS-ELISA. A, Leaf extracts from five healthy *N. tabacum* (A1-A5), five healthy *N. benthamiana* (A6-A10), and wells were treated with buffer (A11 and A12). Leaf extracts from PVX-GUS inoculated *Nt*GFP:TGBp1-101 (C3-G3), *Nt*GFP:CP-107 (C4-G4), *Nt*GFP:TGBp2-102(C5-G5) and *Nt*GFP:GFP-105 (C6-G6),

*Nb*GFP:TGBp1-201 (C7-G7), *Nb*GFP:CP-207 (C8-G8), *Nb*GFP:GFP-201 (C9-G9), nontransgenic *N. benthamiana* (C10-G10), and five positive controls from the kit (C12-G12). B, Leaf extracts from five healthy *N. tabacum* (A1-A5), five healthy *N. benthamiana* (A6-A10), and wells were treated with buffer (A11 and A12). Leaf extracts from PVX-GUS inoculated *Nt*GFP:TGBp1-101 (C3-G3), *Nt*GFP:CP-107 (C4-G4), *Nt*GFP:TGBp2-102(C5-G5) and *Nt*GFP:GFP-105 (C6-G6), *Nb*GFP:TGBp1-201 (C7-G7), *Nb*GFP:CP-207 (C8-G8), *Nb*GFP:GFP-201 (C9-G9), nontransgenic *N. benthamiana* (C10-G10), and five positive controls from the kit (C12-G12). C, Two positive controls from the kit (A1-A2), leaf extracts from mock inoculated *N. tabacum* (B3-B12), *Nt*GFP-108 (C1-G3), *Nt*COI-107(C4-G6), *Nb*GFP:TGBp2-204 (C7-G8), *Nb*GFP-205 (C9-G10), and *Nt*COI-107 (C11-G12) transgenic plants, and mock inoculated *N. benthamiana* (H3-H12).

DISCUSSION

Agrobacterium-mediated genetic transformation is the most preferred and widely used method of gene transfer because of its simplicity, economy, and generation of single-copy transgenic plants (Horsch et al., 1985; Gelvin, 2003). A transformation efficiency of at least 50 % was reported when tobacco cultured cells were co-cultured with *A. tumefaciens* harboring the binary transfer-DNA vector containing a kanamycin resistance marker (An, 1985). There are different methods of screening transgenic plants for the presence of transgene. These include PCR and Southern and Northern blots. PCR is the most commonly used technique to screen putative transformants (Sales et al., 2003). Selecting transgenic plants based on PCR is quick, simple and cost effective as compared to techniques such as Southern or Northern blots because extraction of genomic DNA for PCR is easier than protein extraction for Southern or RNA extraction for Northern analysis. In addition for Southern blots require specific antibody that recognizes a target protein and Northern analysis requires a probe that hybridize with target RNA are needed. Producing antibody or developing a hybridization probe is costly.

Fusing GFP gene to the gene of interest and tracking where and when fusion proteins are made and where they can go is the most popular and widely used technique in molecular biology (Tsien, 1998). The subcellular localization of an uncharacterized protein can be studied using a clone containing the fusion of GFP and an uncharacterized gene and expressing in suitable target cells. The target protein can be monitored using fluorescent microscope to track the subcellular location of the protein. GFP fusions have been routinely used to study virus replication, cell-to-cell and long distance movement, routes for virus movement, and interactions of host and viral factors (Santa Cruz et al.,

1996; Oparka et al., 1997; Imlau et al., 1999; Ryabov et al., 1999; Solovyev et al., 2000; Crawford and Zambryski, 2001; Rojas et al., 2001; Ju et al., 2005; Stadler et al., 2005).

Here we transformed *N. tabacum* and *N. benthamiana* with *A. tumefaciens* strain LBA4404 containing pCOI-GFP:TGBp1, pCOI-GFP:TGBp2, pCOI-GFP:CP, pCOI-GFP:GFP, pCOI-GFP or pCOI plasmids and successfully verified the presence of GFP fused PVX -TGBp1, -TGBp2, -CP encoding ORFs as well as GFP:GFP, GFP and CoYMV in T0 or T1 transgenic lines by PCR. The expression of genes in GFP containing transgenic lines (T0-T2 generations) was verified by the presence of GFP in leaf veins when microscopically examined (Fig. 4). Both PCR and GFP expressions were used as an evidence for the presence and functionality of the fusions in subsequent generations (T0-T2) in both species (Table I, Fig. 2).

β-glucuronidase (GUS) reporter gene tagged PVX infectious clones have been used to study the roles of PVX movement proteins during virus infection (Chapman et al., 1992; Angell et al., 1996; Fedorkin et al., 2000). PVX-GUS was constructed using the full length of PVX cDNA clone (Chapman et al., 1992). First the CP promoter was duplicated then the major portion of CP ORF was deleted leaving only a few bases at the 5' end of CP ORF. The full sequence of GUS was then inserted and next to the GUS gene full sequence of CP ORF was recovered (Chapman et al., 1992). Histochemical detection of GUS activity due to expression of the GUS gene, either in initially infected cells or non inoculated cells, is used as a marker for determining restricted or unlimited virus spread in inoculated plant. In this study, PVX-GUS was used to test susceptibility of transgenic lines. There was a possibility that the transgenic plants generated in this study might be resistant to PVX infection since a previous study reported transgenic

tobacco expressing the PVX CP under the control of 35S promoter was resistant against a broad spectrum of PVX strains (Spillane et al., 1997). Experiments presented here showed all transgenic plants were susceptible for PVX-GUS infection (Table II, Fig 4 & 5). The obvious reason for the opposite result might be that since we used a phloem specific promoter and the fusion genes are exclusively expressed in the vasculature, viral replication in initially infected epidermal cells and other non vascular cells is not affected.

LITERATURES CITED

An G (1985) High efficiency transformation of cultured tobacco cells. Plant Physiol **79:** 568-570

Angell SM, Baulcombe DC (1995) Cell-to-cell movement of *Potato virus X* revealed by micro-injection of a viral vector tagged with the beta-glucuronidase gene. Plant J **7:** 135-140

Angell SM, Davies C, Baulcombe DC (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

Aoki K, Suzui N, Fujimaki S, Dohmae N, Yonekura-Sakakibara K, Fujiwara T, Hayashi H, Yamaya T, Sakakibara H (2005) Destination-selective long-distance movement of phloem proteins. Plant Cell **17:** 1801-1814

Asuka I, Genqing L, Young-Min W, Richard SN, Biao D (2000) Nonspecific intercellular protein trafficking probed by green-fluorescent protein in plants. Protoplasma **213:** 165-175

Balachandran S, Xiang Y, Schobert C, Thompson GA, Lucas WJ (1997) Phloem sap proteins from *Cucurbita maxima* and *Ricinus communis* have the capacity to traffic cell to cell through plasmodesmata. Proc Natl Acad Sci U S A **94:** 14150-14155

Baluska F, Cvrckova F, Kendrick-Jones J, Volkmann D (2001) Sink Plasmodesmata as gateways for phloem unloading. myosin viii and calreticulin as molecular determinants of sink strength? Plant Physiol. **126:** 39-46

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

Carrington JC, Kasschau KD, Mahajan SK, Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell **8:** 1669-1681

Chapman S, Kavanagh T, Baulcombe D (1992) *Potato virus X* as a vector for gene expression in plants. Plant J **2:** 549-557

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

Ding B, Haudenshield JS, Hull RJ, Wolf S, Beachy RN, Lucas WJ (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 X, Shintaku MH, Carter SA, Nelson RS (1996) Invasion of minor veins of tobacco leaves inoculated with *Tobacco mosaic virus* mutants defective in phloem-dependent movement. Proc Natl Acad Sci USA **93:** 11155-11160

Fedorkin ON, Merits A, Lucchesi J, Solovyev AG, Saarma M, Morozov SY, Makinen K (2000) Complementation of the movement-deficient mutations in *Potato virus X*: potyvirus coat protein mediates cell-to-cell trafficking of C-terminal truncation but not deletion mutant of potexvirus coat protein. Virology **270:** 31-42

Gelvin SB (2003) Agrobacterium-mediated plant transformation: the biology behind the "Gene-Jockeying" tool. Microbiol. Mol. Biol. Rev. **67:** 16-37

Gittins JR, Pellny TK, Biricolti S, Hiles ER, Passey AJ, James DJ (2003) Transgene expression in the vegetative tissues of apple driven by the vascular-specific rolC and CoYMV promoters. Transgenic Res **12:** 391-402

Golecki B, Schulz A, Thompson GA (1999) Translocation of structural P proteins in the phloem. Plant Cell **11:** 127-140

Gomez G, Pallas V (2004) A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex in vivo with *Hop Stunt viroid* RNA. J. Virol. **78:** 10104-10110

Horsch RB, Fry JE, Hoffmann NL, Eicholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227:** 1229-1231

Howard AR, Heppler ML, Ju HJ, Krishnamurthy K, Payton ME, Verchot-Lubicz J (2004) *Potato virus X* TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves. Virology **328:** 185-197

Imlau A, Truernit E, 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, Ma F, Qi Y, Matsuda Y, Zhu Y, Liang G, Ding B (2002) Plasmodesma-mediated selective protein traffic between "symplasmically isolated" cells probed by a viral movement protein. Plant Cell **14:** 2071-2083

Ju HJ, Brown JE, Ye CM, Verchot-Lubicz J (2007) Mutations in the central domain of *Potato virus X* TGBp2 eliminate granular vesicles and virus cell-to-cell trafficking. J Virol **81:** 1899-1911

Ju HJ, Samuels TD, Wang YS, Blancaflor E, Payton M, Mitra R, Krishnamurthy K, Nelson RS, Verchot-Lubicz J (2005) The *Potato virus X* TGBp2 movement protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol **138:** 1877-1895

Krishnamurthy K, Heppler M, Mitra R, Blancaflor E, Payton M, Nelson RS, 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

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

Lough TJ, Emerson SJ, Lucas WJ, Forster RL (2001) Trans-complementation of long-distance movement of *White clover mosaic virus* triple gene block (TGB) mutants: phloem-associated movement of TGBp1. Virology **288:** 18-28

Lough TJ, Netzler NA, Emerson SJ, Sutherland P, Carr F, Beck DL, Lucas WJ, Forster RL (2000) Cell-to-cell movement of potexviruses: evidence for a ribonucleic protein complex involving the coat protein and first triple gene block protein. Mol Plant Microbe Interact **13:** 13:962–974

Lucas WJ, Wolf S (1999) Connections between virus movement, macromolecular signaling and assimilate allocation. Curr Opin Plant Biol **2:** 192-197

Matsuda Y, Liang G, Zhu Y, Ma F, Nelson RS, Ding B (2002) The *Commelina yellow mottle virus* promoter drives companion-cell-specific gene expression in multiple organs of transgenic tobacco. Protoplasma **220:** 51-58

Medberry SL, Lockhart BE, Olszewski NE (1992) The *Commelina yellow mottle virus* promoter is a strong promoter in vascular and reproductive tissues. Plant Cell **4:** 185-192

Medberry SL, Olszewski NE (1993) Identification of cis elements involved in *Commelina yellow mottle virus* promoter activity. Plant J **3:** 619-626

Mezitt LA, Lucas WJ (1996) Plasmodesmal cell-to-cell transport of proteins and nucleic acids. Plant Mol Biol **32:** 251-273

Mitra R, Krishnamurthy K, Blancaflor E, Payton M, Nelson RS, 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:** 35-48

Oparka KJ (1990) What is phloem unloading? Plant Physiol **94:** 393-396

Oparka KJ, Prior DA, Santa Cruz S, Padgett HS, Beachy RN (1997) Gating of epidermal plasmodesmata is restricted to the leading edge of expanding infection sites of *Tobacco mosaic virus* (TMV). Plant J **12:** 781-789

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

Oparka KJ, Santa Cruz S (2000) THE GREAT ESCAPE: Phloem transport and unloading of macromolecules. Annu Rev Plant Physiol Plant Mol Biol **51:** 323-347

Rojas MR, Jiang H, Salati R, Xoconostle-Cazares B, Sudarshana MR, Lucas WJ, Gilbertson RL (2001) Functional analysis of proteins involved in movement of the monopartite begomovirus, *Tomato yellow leaf curl virus*. Virology **291:** 110-125

Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. Development **126:** 4405-4419

Ruiz-Medrano R, Xoconostle-Cazares B, Lucas WJ (2001) The phloem as a conduit for inter-organ communication. Curr Opin Plant Biol **4:** 202-209

Ryabov EV, Oparka KJ, Santa Cruz S, Robinson DJ, Taliansky ME (1998) Intracellular location of two *Groundnut rosette umbravirus* proteins delivered by PVX and TMV vectors. Virology **242:** 303-313

Ryabov EV, Roberts IM, Palukaitis P, Taliansky M (1999) Host-specific cell-to-cell and long-distance movements of *Cucumber mosaic virus* are facilitated by the movement protein of groundnut rosette virus. Virology **260:** 98-108

Sales E, Segura J, Arrillaga I (2003) *Agrobacterium tumefaciens*-mediated genetic transformation of the cardenolide-producing plant *Digitalis minor* L. Planta Medica**:** 143-147

Santa Cruz S, Chapman S, Roberts AG, Roberts IM, Prior DA, Oparka KJ (1996) Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. Proc Natl Acad Sci U S A **93:** 6286-6290

Santa Cruz S, Roberts AG, Prior DA, Chapman S, Oparka KJ (1998) Cell-to-cell and phloem-mediated transport of *Potato virus X*. The role of virions. Plant Cell **10:** 495-510

Santa Cruz S, Roberts AG, Prior DAM, Chapman S, Oparka KJ (1998) Cell-to-Cell and Phloem-Mediated Transport of *Potato virus X*: The Role of Virions. Plant Cell **10:** 495-510

Solovyev AG, Stroganova TA, Zamyatnin AA, Jr., Fedorkin ON, Schiemann J, Morozov SY (2000) Subcellular sorting of small membrane-associated triple gene block proteins: TGBp3-assisted targeting of TGBp2. Virology **269:** 113-127

Spillane C, Verchot J, Kavanagh TA, Baulcombe DC (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:** 76-84

Stadler R, Wright KM, Lauterbach C, Amon G, Gahrtz M, Feuerstein A, Oparka KJ, Sauer N (2005) Expression of GFP-fusions in Arabidopsis companion cells reveals non-specific protein trafficking into sieve elements and identifies a novel post-phloem domain in roots. Plant J **41:** 319-331

Tamai A, 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. Mol Plant Microbe Interact **14:** 1158-1167

Tsien RY (1998) The green fluorescent protein. Annu Rev Biochem **67:** 509-544

Turgeon R, Medville R, Nixon KC (2001) The evolution of minor vein phloem and phloem loading. Am. J. Bot. **88:** 1331-1339

Van Bel AJE (1993) Strategies of phloem loading. Annu Revi Plant Physiol Plant Mole Biol **44:** 253-281

Voitsekhovskaja OV, Koroleva OA, Batashev DR, Knop C, Tomos AD, Gamalei YV, Heldt HW, Lohaus G (2006) Phloem loading in two Scrophulariaceae species. What can drive symplastic flow via plasmodesmata? Plant Physiol **140:** 383-395

Wintermantel WM, Banerjee N, Oliver JC, Paolillo DJ, Zaitlin M (1997) *Cucumber mosaic virus* is restricted from entering minor veins in transgenic tobacco exhibiting replicase-mediated resistance. Virology **231:** 248-257

Xoconostle C, aacute, zares B, Xiang Y, Ruiz-Medrano R, Wang H-L, Monzer J, Yoo B-C, McFarland KC, Franceschi VR, Lucas WJ (1999) Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. Science **283:** 94-98

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

Zhang X-Y, Wang X-L, Wang X-F, Xia G-H, Pan Q-H, Fan R-C, Wu F-Q, Yu X-C, Zhang D-P (2006) A shift of phloem unloading from symplasmic to apoplasmic pathway is involved in developmental onset of ripening in grape berry. Plant Physiol. **142:** 220-232

Zhu Y, Qi Y, Xun Y, Owens R, Ding B (2002) Movement of *Potato spindle tuber viroid* reveals regulatory points of phloem-mediated RNA traffic. Plant Physiol. **130:** 138-146

CHAPTER III

PHLOEM UNLOADING OF PVX PROTEINS IN TRANSGENICALLY EXPRESSED COMPANION CELLS

ABSTRACT

Experiments were conducted using the transgenic plants generated in Chapter II to determine the requirements for exit of the PVX TGBp1, TGBp2, and CP from companion cells. Leaves, petioles and stem sections from non-infected and PVX-GUS infected transgenic plants were excised from live plants and viewed using epifluorescence microscopy to characterize the pattern of GFP movement out of companion cells. For noninfected transgenic leaves, fluorescence was primarily restricted to the phloem and did not appear in cells surrounding the veins. However, in PVX-GUS infected *Nb*GFP:TGBp1-201 and *Nb*GFP:CP-207 fluorescence was seen in neighboring cells of class III, IV and V veins. In non-infected petiole cross sections, only GFP:TGBp1 was seen in xylem parenchyma, but in PVX-GUS infected petioles, fluorescence spread into cells surrounding the xylem parenchyma. In non-infected *Nb*GFP:TGBp1-201 stem cross sections, fluorescence was seen in the xylem parenchyma while in PVX-GUS infected stems, fluorescence spread into the pith. Petiole segments of non-infected *Nb*GFP:TGBp1-201 transgenic plants were embedded in LR-White and cross sections were immunolabeled with PVX TGBp1 antisera. The results of immunolabelling showed that GFP:TGBp1 accumulates in the companion cells, sieve

elements, phloem parenchyma, xylem parenchyma, pericycle, and endodermis. Nontransgenic scions were grafted onto transgenic root stocks and each of the fusions traversed the graft union junction. 5 (6)-Carboxyfluorescein (CF) dye and PVX-GUS were separately applied to the root stocks and translocated through graft union unloading in sink leaves.

INTRODUCTION

Plant proteins, nucleic acids, and other macromolecules contributing to growth, differentiation, and defense are translocated over long distances via the phloem (Oparka and Santa Cruz, 2000). Macromolecules often exit the phloem and move between cells through plasmodesmata to reach target tissues (Lucas and Wolf, 1999; Oparka and Santa Cruz, 2000). Stems contains two rings of internal and external phloem with numerous tracheary elements and xylem parenchyma cells forming a bridge between the two rings (Esau, 1965; Wu et al., 2002). The internal and external phloem extends from the stem into the leaf petiole, which Avery (1933) defined as the "vascular arc". The vascular arc has alternating rows of xylem vessels and xylem parenchyma with strands of internal and external phloem, pericycle, and endodermis on each side of the xylem (Fig. 5B).

In *N. tabacum* and *N. benthamiana,* leaves contain a network of veins classified as class I-V and grouped into major (class I-III veins) or minor (class IV and V veins) veins according to their structural complexity, position, and function (Roberts et al., 1997; Nelson and van Bel, 1998; Cheng et al., 2000). The internal phloem extends from the petiole into the midrib which is also defined as class I veins. Additional lateral veins extend toward the leaf lamina. Class II veins branches from class I veins, class III veins branch from class II veins, class IV veins branch from class III veins, and class V veins are branches from class IV veins.

Class III veins can have approximately 5-6 sieve elements, companion cells, and phloem parenchyma and an undefined number of bundle sheath cells. In class III veins, plasmodesmata connect sieve elements, companion cells, phloem parenchyma, and bundle sheath cells (Roberts et al., 1997). Plasmodesmata also connect bundle sheath

cells to surrounding mesophyll cells providing a path for viruses and other macromolecules to exit from the phloem into surrounding leaf mesophyll in sink tissues (Roberts et al., 1997). For example, experiments using PVX-GFP, *Cowpea mosaic virus* (CPMV) phloem, or the phloem-translocatable carboxyfluorescein (CF) dye, showed virus and CF dye unloading from class III veins into mesophyll cells in sink leaves. As leaves age and transition from photosynthetic sinks to sources, CF-dye as well as the viruses were no longer able to unload from class III veins into neighboring tissues suggesting that the direction of flow in class III veins is reversed during maturation. Plasmodesmata connections between class III vein cells and bundle sheath cells may also be down regulated reducing the export of macromolecules into neighboring mesophyll cells (Roberts et al., 1997; Cheng et al., 2000; Oparka and Santa Cruz, 2000; Silva et al., 2002).

Class IV and V veins consist of two or three sieve elements, companion cells, and phloem parenchyma, and bundle sheath cells (Oparka and Santa Cruz, 2000). In sink leaves, class IV and V veins are not fully differentiated and are symplastically connected to the mesophyll. As the leaf matures and transitions from sink to source, the sieve elements and companion cells fully develop. Thus in sink leaves, class IV and V veins are not functional for phloem loading or unloading, but in mature leaves class IV and V veins provide a path for entry of viruses and macromolecules into the vasculature (Esau, 1965; Oparka and Santa Cruz, 2000).

Three models have been proposed in separate studies to explain the translocation and exit of macromolecules from the phloem into target tissues. Target tissues are sometimes sink tissues but may more generally be described as distant tissues which

require proteins, nucleic acids, or macromolecules to facilitate growth, metabolism or defense. It is reasonable to consider that virus translocation and exit from the phloem may function similarly to these models. The first model is the "destination selective" model, in which cellular chaperones function to guide proteins or RNAs towards their target tissues (Aoki et al., 2005). According to this model, proteins or RNAs are prevented from accumulating in nontarget tissues by the cells' degradation machinery. One such chaperone is called CmPP16 which is a homolog of dianthoviral movement protein (Xoconostle-Cazares et al., 1999), has RNA binding activity, and is preferentially unloaded in root tissues. CmPP16 is degraded in nontarget tissues (Aoki et al., 2002). The second model is the "relay race" model which was used to describe translocation of RNA silencing signals and plant RNA viruses. This model requires polymerase functions to amplify nucleic acids as they move from cell-to-cell. Thus the RNA silencing signal or virus is not diluted as it spreads and high titers can be maintained for long distance transport. Continual amplification of the nucleic acids is important to compete with the cells' degradation machinery which may act to reduce spread of the nucleic acids (Voinnet et al., 1998). The third model for nucleic acid transport is the "RNP model". Free or naked viral RNA might be vulnerable to degradation by the host, thus formation of a RNP complex is required for translocation and exit from phloem(Blackman et al., 1998; Lough et al., 2000; Verchot, 2005; Karpova et al., 2006).

Each of the three models indicate that the exit of macromolecules from the veins may be negatively regulated by cellular degradation machinery. We know that the 26S proteasome regulates TMV MP accumulation and virus cell-to-cell movement (Reichel

and Beachy, 2000). Thus it is reasonable to assume that the host degradation machinery may determine selective transport of viruses into target tissues.

Potato virus X (PVX) encodes the TGBp1, TGBp2, TGBp3 and coat (CP) proteins which are required for virus movement between adjacent cells and through the vasculature (Lough et al., 2000; Lough et al., 2001; Lough et al., 2006). A model was proposed suggesting that TGBp1 and CP form a ribonucleoprotein complex which traffics viral RNA from cell-to-cell and through the vasculature (Lough et al., 2000). TGBp1 also functions as a suppressor of RNA silencing and has the ability to move from cell-to-cell through plasmodesmata. PVX TGBp2 and TGBp3 are associated with the ER (Tamai and Meshi, 2001; Krishnamurthy et al., 2003; Ju et al., 2005; Ju et al., 2007).

In this study we used transgenic plants expressing GFP fused to the PVX TGBp1, TGBp2, and CP genes to study protein and virus exit from the phloem. The fused genes were transgenically expressed in phloem companion cells from a CoYMV promoter. In this study we found that the three fusion proteins were restricted primarily to phloem in the leaves. However GFP:TGBp1 was seen to exit the phloem into surrounding tissues in the petiole and stem. When transgenic plants were infected with PVX, minor amounts of GFP:TGBp1 and GFP:CP were seen in cells surrounding class III, IV and V veins. Grafting experiments showed that the transgenically expressed proteins were phloem mobile. These results indicate the PVX proteins exit the phloem in a manner that is highly regulated and depends on viral as well as host factors.

MATERIALS AND METHODS

Plant Material, Virus Inoculations, and Epifluorescence Microscopy

In this studies nontransgenic *Nicotiana benthamiana,* nontransgenic *N. tabacum,* *Nt*GFP:TGBp1-101, *Nt*GFP:TGBp2-102, *Nt*GFP:CP-117, *Nt*GFP:GFP-105, *Nt*GFP-108 , *Nb*GFP:TGBp1-201, *Nb*GFP:TGBp2-204, *Nb*GFP:CP-207, *Nb*GFP:GFP-201, *Nb*GFP-205 lines were used.

Nontransgenic and transgenic plants were inoculated with PVX-GUS transcripts or infected plant sap (Chapman et al., 1992; Baulcombe et al., 1995). The mMessage mMachine T7 kit (Ambion Inc., Austin, TX) was used to prepare infectious PVX-GUS transcripts. One μg *Spe*I linearized plasmid was added to each transcription reaction. Transcription reactions were directly used to inoculate plants in the greenhouse. Leaves were dusted with carborundum and then rub inoculated with 2-5 μL (between 30-150 μg) transcript (Chapman et al., 1992; Baulcombe et al., 1995). Following inoculation, plants were misted with water. For some experiments, PVX-GUS infected *N. benthamiana* leaves were excised and ground in a mortar and pestle with 2 volume (w/v) 0.1 M phosphate buffer, pH 8.0 (94 mL of 1 M K_2HPO_4, 6 mL of 1M KH_2PO_4, plus 900 mL ddH_2O) plus 0.2% β-mercaptoethanol (v/v) and 10% ethanol (v/v) and used for rub inoculation as described above for transcript. Transgenic and non transgenic *N. benthamiana* plants were analyzed one week after inoculation, but transgenic and non transgenic *N. tabacum* were analyzed two weeks after inoculation.

Leaves, stems, and petioles from inoculated and noninoculated samples were analyzed for expression of GFP using epifluorescence microscopy. Transverse sections of petioles and stems were obtained using a razor blade and then mounted onto glass

slides. GFP fluorescence was analyzed using a Nikon E600 epifluorescence microscope with a Nikon B2A filter cube (containing a 470- to 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.) attached to the Nikon E600 microscope. All images obtained by epifluorescent microscopy, were processed using Adobe Photoshop CS version 8.0 software (Adobe Systems, San Jose, CA). Sections were scored for the presence of fluorescence in the companion cells, sieve elements, vascular parenchyma, bundle sheath cells, and mesophyll cells.

Fixation, Embedding, and Immunolabelling of Petiole Segments

Petiole cross sections from *Nb*GFP:TGBp1-201, *Nb*GFP:CP-207, *Nb*COI-203 and uninfected and PVX-GUS infected *N. benthamiana* plants were fixed for 2 h at room temperature under vacuum in a 10 ml solution containing 0.5% (v/v) glutaraldehyde (v/v), 4.0% paraformaldehyde (v/v), and 34.2 g L^{-1} sucrose in 50 mM sodium cacodylate buffer (v/v) (pH 7.2)(Driskel et al., 2004). The samples were embedding in LR White resin (Ted Pella, Redding, CA). Thick sections (500 nm) were cut using a glass knife on a Sorvall MT 6000 ultramicrotome and mounted on ProbeOn Plus slides (Fisher Biotechnology, Pittsburgh, PA)(Driskel et al., 2004). Sections were stained with Toluidin blue (0.1% in H_2O).

For immunolabelling, sections were incubated with blocking solution (20 g L^{-1} bovine serum albumin (w/v) (BSA) fraction V in 6.06 g L^{-1} Tris-HCl, pH 7.4, 8.77 g L^{-1} NaCl) at room temperature for 1 h. *Nb*GFP:TGp1-201 samples were then treated with goat anti-TGBp1 sera (1:128) prepared by Dr Chang-Ming Ye, and *Nb*GFP:CP-207 was

treated with rabbit anti-PVX polyclonal antisera (1:100 and 1:200) obtained from Agdia in antisera diluents (6.06 g L^{-1} Tris-HCl (pH 7.4), 8.77 g L^{-1} NaCl, and 1 g L^{-1} BSA), or buffer and incubated over night at 4 ^{0}C in a moist box. Samples were washed 4 times 5 min each with blocking solution at room temperature. Either Alexa Fluor® 488 IgG rabbit anti-goat (Invitrogen Corp., Carlsbad, CA) diluted 1:100 to 1:200 (v/v) or Alexa Fluor® 488 goat anti-rabbit diluted 1:100 to 1:200 (v/v) was used as secondary antisera for TGBp1 or CP, respectively. Slides were incubated at room temperature for 1-2 h in a dark moist plastic box and then washed twice with blocking buffer and one time with filtered distilled water, each for five min. To prevent rapid loss of fluorescence during microscopic examination VECTASHIELD® Mounting Medium (Vector Laboratories**,** Burlingame, CA) was applied and a microcover slip was fixed into place with nail polish. A Leica TCS SP2 (Leica Microsystem, Bannockburn, IL) confocal imaging system was used to study the presence or absence of signal in companion cells, sieve element, phloem parenchyma, xylem parenchyma, xylem, pericycles and endodermis. The Leica TCS SP2 system is attached to a Leica DMRE microscope (Leica Microsystems). For GFP expression, 488 nm excitation wavelength produced by Krypton/Argon lasers were used. All images obtained by confocal, were processed using Adobe Photoshop CS version 8.0 software (Adobe Systems, San Jose, CA).

Side Grafting and CF Dye Loading

Non transgenic scions *N. tabacum* were grafted onto transgenic rootstocks. Scions carrying two leaves between 0.5 and 1 cm were taken from the tips of non transgenic plants and a short smooth edge were made, stuck into the stem at a slant

cutting made vertically down to make opening and the scion was inserted at the side and then both rootstock portion above the cutting and scion were wrapped with tape (Fig. 5). To avoid desiccation, grafted plants were kept under a white plastic bag for 3 to 5 days in a plastic humidity chamber. Ten to 15 days after grafting the portion of the rootstock above the graft union was removed. Plants were analyzed 2-4 weeks after grafting. Stem segments were cut with a razor blade starting from 0.5 cm above the interface of graft union and analyzed by epifluorescence microscopy. Sections were photographed and scored for the presence of fluorescence in the companion cells, sieve elements, vascular parenchyma, pith, and mesophyll cells. In addition, nontransgenic scions were grafted onto nontransgenic rootstocks infected with PVX-GUS and analyzed for spread of the virus from root stock into leaves of scion. 5(6)-carboxyfluorescein diacetate (Molecular Probes) solution of 6 mg mL^{-1} stock in acetone was prepared and diluted to 0.24 mg mL^{-1} in water (Kathryn and Oparka, 1996). The source leaf was cut leaving 1cm petiole on the stem and an eppendorf tube with cutting at bottom was stuck to petiole and the bottom of the tube was sealed with parafilm. CF dye (1mL) solution was added into the tube and the cap was closed. Then scions were analyzed with microscope six h after CF dye application.

Statistical Analysis

All quantitative data collected in this study were processed either with Excel or SAS 9.1 software package by Mark Payton.

The number of cell types within phloem and surrounding tissues were scored for the presence or absence of fluorescent signal from two fields containing companion cells,

sieve elements, phloem parenchyma, xylem parenchyma, and pericycles in each section (80 fields from NbGFP:TGBp1-201 and 100 fields from NbGFP:CP-207) and subjected for analysis. ANOVA procedures with PC SAS version 9.1 (SAS Institute, Cary, NC) and PROC GLM were used in Table II to compare the presence of fluorescent signal in petiole cross sections of *Nb*GFP:TGBp1-201 or *Nb*GFP:CP-207 within phloem and surrounding tissues treated with TGBp1 or CP antisera (Table II). Comparisons were made between TGBp1 or CP antisera treated *Nb*GFP:TGBp1-201 or *Nb*GFP:CP-207 and PVX-GUS infected *N. benthamiana* and non-infected *N. benthamiana*. For all above analysis when ANOVA was significant, pairwise comparisons of means were made with a PDIFF option in an LSMEANS statement. A significant level of 0.01 was used for all comparisons.

RESULTS

Fusion Proteins are Restricted mainly to the Phloem

Selected transgenic tobacco lines are listed in Table I and are named according to the transgene introduced: GFP:TGBp1, GFP:TGBp2, GFP:CP, GFP:GFP, or GFP. The line number is indicated after the name of the transgene and these specific T2 lines were selected because they showed high levels of fluorescence in the vasculature and were determined to be susceptible to virus infection (see Chapter II). Since plasmodesmata connect companion cells with sieve elements and phloem parenchyma, we predict that proteins expressed in the companion cells may move into these neighboring cells. Since TGBp1, TGBp2, and CP are required for virus cell-to-cell and vascular transport, it is highly likely that these fusion proteins should accumulate outside the companion cells. Live segments of leaves, petioles and stems were initially viewed under an epifluorescence microscope to determine if the GFP:TGBp1, GFP:TGBp2, GFP:CP, and GFP:GFP fusion proteins spread beyond the companion cells into neighboring cells. Evidence of fluorescence in vascular cells surrounding the companion cells would indicate that the proteins can move through symplastic connections from companion cells into neighboring vascular cells. Evidence that GFP:TGBp1, GFP:TGBp2, or GFP:CP selectively unloads into nonvascular cells in specific tissues would indicate tissue trophism or support the destination selective model for proteins exiting the phloem. Finally, evidence that the accumulation pattern of the PVX fusion proteins differs from GFP:GFP would indicate selective transport of the viral proteins.

Table I. *Microscopic analysis of GFP expression in leaf veins, petiole and stem in the presence and absence of PVX-GUS infection*

Fifteen healthy and PVX-GUS infected plants were analyzed for protein unloading from leaf veins (Class I-V) into surrounding mesophyll or epidermal tissues. Leaf surfaces and cross sections through the veins were analyzed using epifluorescent microscopy.

		No. of plants showing protein unloading from						
				veins[a]				
Transgenic lines	Total No. Plants	Petiole[b]	Stem[b]	I	II	III	IV	V
Healthy Plants								
*Nt*GFP:TGBp1-101	15	0	0	0	0	0	0	0
*Nt*GFP:TGBp2-102	15	0	0	0	0	0	0	0
*Nt*GFP:CP-117	15	0	0	0	0	0	0	0
*Nt*GFP:GFP-105	15	0	0	0	0	0	0	0
*Nt*GFP-108	15	0	0	0	0	0	0	0
*Nb*GFP:TGBp1-201	15	2	2	0	0	0	0	0
*Nb*GFP:TGBp2-204	15	0	0	0	0	0	0	0
*Nb*GFP:CP-207	15	0	0	0	0	0	0	0
*Nb*GFP:GFP-201	15	0	0	0	0	0	0	0
*Nb*GFP-205	15	0	0	0	0	0	0	0
PVX-GUS infected plants								
*Nt*GFP:TGBp1-101	15	0	0	0	0	0	0	0
*Nt*GFP:TGBp2-102	15	0	0	0	0	0	0	0
*Nt*GFP:CP-117	15	0	0	0	0	0	0	0
*Nt*GFP:GFP-105	15	0	0	0	0	0	0	0
*Nt*GFP-108	15	0	0	0	0	0	0	0
*Nb*GFP:TGBp1-201	18	2	2	0	0	3	5	5
*Nb*GFP:TGBp2-204	15	0	0	0	0	0	0	0
*Nb*GFP:CP-207	18	0	0	0	0	3	5	5
*Nb*GFP:GFP-201	18	0	0	0	0	0	0	0
*Nb*GFP-205	15	0	0	0	0	0	0	0

[a] Vein classes are indicated as I-V. The total numbers of plants showing fluorescence in mesophyll or epidermal cells surrounding each vein. The plant designation *Nt* or *Nb* refers to *Nicotiana tabacum* or *N. benthamiana*, followed by the name of the transgene. The -100 and -200 numbers refer to individual lines selected for further analysis.
[b] number of plants were scored as positive if fluorescence was seen in the xylem parenchyma and pericycle

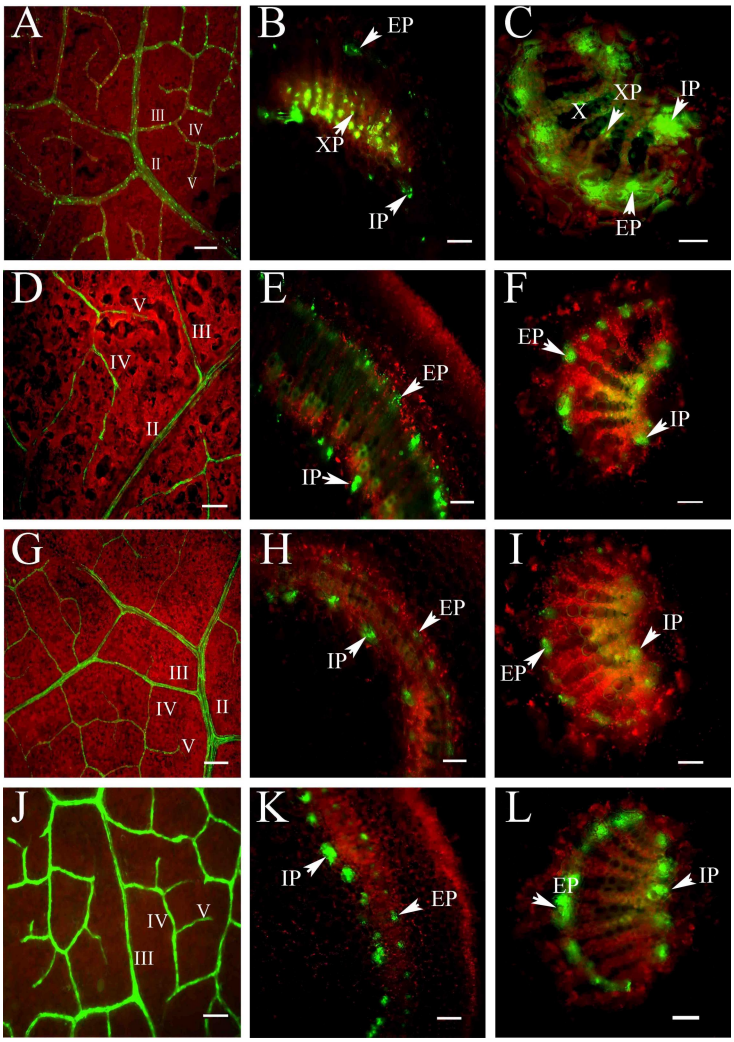


Figure 1. Each row shows representative epifluorescence images of a leaf surface, stem cross sections, and petiole cross sections, respectively. Images taken from plants expressing the transgene: *Nt*GFP:TGBp1-101 (A), *Nb*GFP:TGBp1-201(B, C), *Nt*GFP:TGBp2-102 (D), *Nb*GFP:TGBp2-204 (E, F), *Nt*GFP:CP-117 (G), *Nb*GFP:CP-207(H, I), and *Nt*GFP:GFP-105 (J), *Nb*GFP:GFP-201 (K, L). Roman numbers in leaf images identify vein classes. A, *Nt*GFP:TGBp1 leaf; B and C, cross sections of stem and petioles of *Nb*GFP:TGBp1 show TGBp1 unloading from phloem. EP, External phloem; IP, Internal phloem; XP, Xylem parenchyma; X, Xylem. Bars represent100μM.

Transgenes were Expressed in Companion Cells

In cross sections of *Nb*GFP:TGBp1-201 (Fig 1B and C), *Nb*GFP:TGBp2-204 (Fig 1E and F), and *Nb*GFP:CP-207 (Fig 1H and I) and *Nb*GFP:GFP-201 (Fig 1K and L) transgenic plant petioles and stems we observed fluorescence throughout the phloem. Fluorescence was not restricted to single companion cells. Hand sections provide low resolution evidence that proteins are likely in many cells associated with the phloem (companion cells, sieve elements, phloem parenchyma). We scored 30 stems and petioles for the occurrence of fluorescence in tissues surrounding the phloem strands.

Leaf segments were viewed under the microscope to study each vein class. Veins are classified in to five based on branching pattern (Avery, 1933; Roberts et al., 1997). Midrib of leaf is class I vein. The midrib (class I vein) gives rise at regular intervals to class II veins. The class III veins originate from class II veins and from a network in the leaf lamina. Veins branching with in the network of class III veins form class IV veins. Veins further branching from class IV veins and with dead end are termed class V veins. Previous studies of PVX, Barley stripe mosaic virus (BSMV), CPMV (Roberts et al., 1997; Haupt et al., 2001; Silva et al., 2002) showed that virus typically exits class III veins (but not class IV or class V veins) into the mesophyll.

In transgenic plants expressing GFP:TGBp1 (Fig 1A), GFP:TGBp2 (Fig 1D), GFP:CP (Fig 1G), or GFP:GFP (Fig 1J), fluorescence was seen in leaf vein classes I-V. Fluorescence was not seen in surrounding mesophyll tissues indicating that the proteins were restricted to the phloem. Leaves from 15 plants were scored and none showed fluorescence in surrounding mesophyll or epidermal cells (Table I).

We also scored 15 plants (Table I) for the occurrence of fluorescence in tissues surrounding the leaf petiole, and stem. In 15% of the transgenic *Nb*GFP:TGBp1-201 plants analyzed, fluorescence spread from the internal phloem into neighboring xylem parenchyma cells in the stem and petiole (Table I; Fig. 1B and E). GFP:TGBp2, GFP:CP, and GFP:GFP were restricted to the phloem and did not appear to spread into neighboring mesophyll or epidermal cells (Table 1). These data indicate that GFP:TGBp1 has the specific ability to move beyond the phloem into neighboring cells in the petiole and stem but not in leaves.

Since GFP:TGBp1 can exit the phloem into surrounding tissues in the stem and petiole but is not seen to exit the phloem in leaf veins, it is likely that the bundle sheath cells provide a barrier for virus export into cells neighboring the veins in tobacco leaves. It is also reasonable to hypothesize that different cellular mechanisms regulate phloem exit into nonvascular tissues in the leaves versus the stems, and petioles.

PVX Selectively Aids Exit of GFP:TGBp1 and GFP:CP From the Phloem in Leaves

To determine if PVX infection alters the pattern of fluorescence in plants, transgenic plants were inoculated with PVX-GUS. Leaf veins, stems, and petioles of systemically infected plants were microscopically examined for fluorescence in the surrounding nonvascular tissues between 5 and 10 days post-inoculation (Table I). At 6 days post-inoculation, we first detected fluorescence in cells neighboring class III, IV and V veins

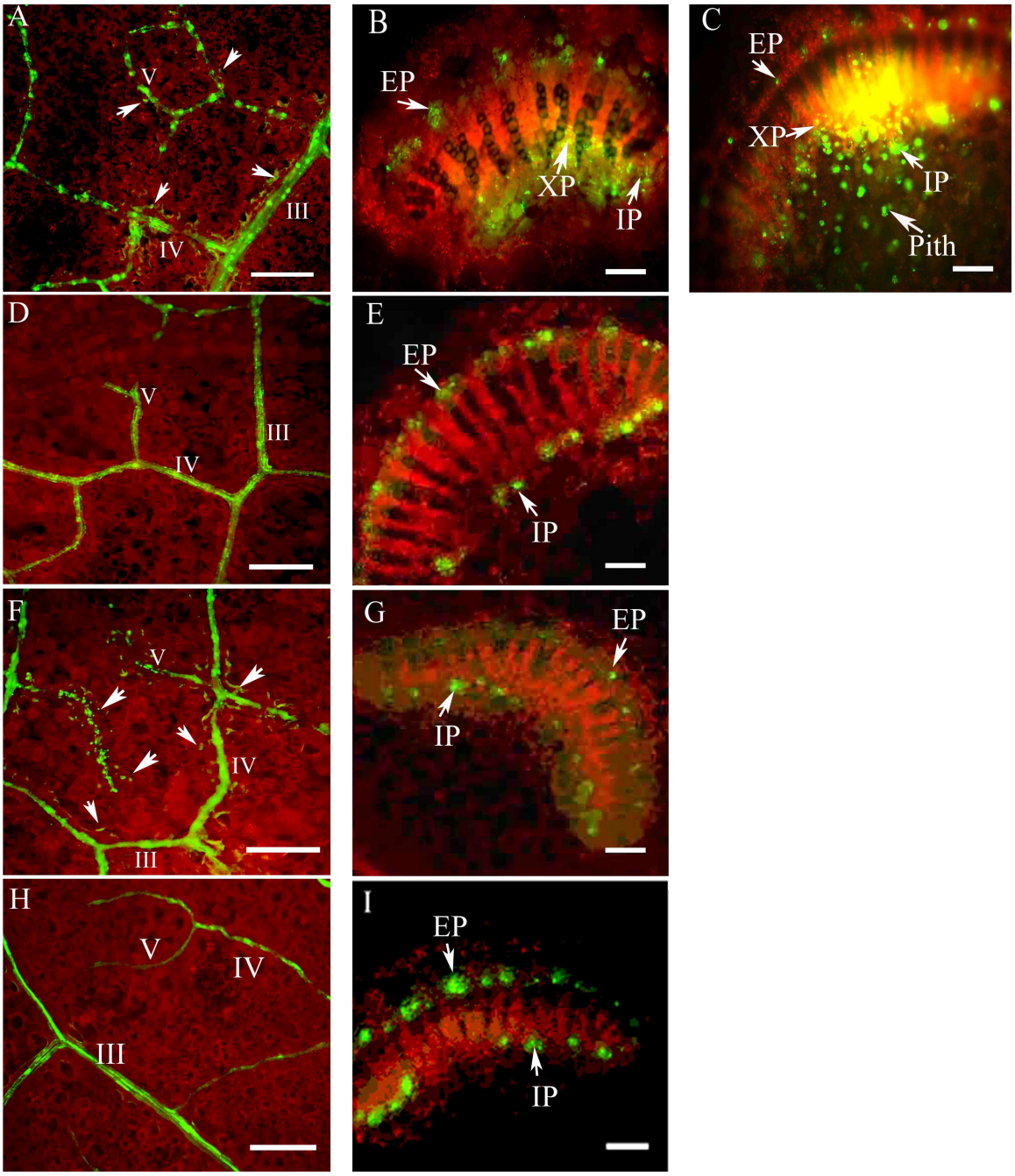


Figure 2. Epifluorescence images showing localization of GFP:TGBp1, GFP:TGBp2, GFP:CP and GFP:GFP fusion proteins in leaf veins, petiole and stem cross sections of PVX-GUS infected transgenic *N. benthamiana*. A, B and C, Images of *Nb*GFP:TGBp1-201 leaf veins, petiole, and stem, respectively. D and E, Images of *Nb*GFP:TGBp2-204 leaf veins and petiole, respectively. F and G, Images of *Nb*GFP:CP-207 leaf veins and

petiole, respectively. H and I, Image of *Nb*GFP:GFP-201 leaf veins and petiole, respectively. Roman numbers show vein classes. Arrows in A and F indicates protein unloading to neighboring cells from veins. Arrows in B and C indicates protein unloading to xylem parenchyma (XP) and pith from phloem. EP, External phloem; IP, Internal phloem. Bars represent 100μM

in the upper leaves of *Nb*GFP:TGBp1-201 and *Nb*GFP:CP-207 (Fig. 2A and F) plants. These data indicate PVX either promoted protein exit from the veins or increased protein accumulation to detectable levels in surrounding tissues.

PVX-GUS had no impact on the numbers of plants showing GFP:TGBp1 unloading in the stem and petiole but the virus did alter the extent of protein movement into surrounding tissues in the stem (Fig. 2C). In cross sections of virus infected stems, GFP:TGBp1 fluorescence was seen in the xylem parenchyma and pith (Fig. 2C). These data suggest that extensive accumulation of GFP:TGBp1 outside of the phloem requires the presence of PVX.

Immunofluorescent Labeling Shows GFP:TGBp1 and GFP:CP Moves out of Phloem

The cross sections of stem, and petioles showed fluorescence spread beyond the companion cells for most transgenic lines. We embedded petiole segments and conducted immunofluorescence labeling to determine the extent of protein accumulation outside of the companion cells. While we lack antisera to GFP and TGBp2, immunofluorescence labeling using PVX TGBp1 and CP antiserum was conducted to determine if the related fusion proteins accumulate outside of the phloem at levels that may be lower than detectable using hand sections of intact tissues. Since the petiole provides bidirectional (Trip and Gorham, 1968) communication between the leaf and stem, experiments using petioles will provide the best opportunity to view protein exit from the phloem irrespective of the direction of bulk flow translocation. Petiole segments from PVX-GUS infected and healthy nontransgenic, *Nb*GFP:TGBp1-201,

*Nb*GFP:CP-207 transgenic plants were embedded in LR white and thick sections were prepared for immunolabelling. Samples were treated with Alexaflour 488 antisera and confocal microscopy was used to determine the subcellular distribution of the transgenically expressed proteins (Table II, Fig. 3).

Sections were scored for the presence of fluorescence in the vasculature and surrounding tissues: internal/external companion cells, internal/external sieve elements, internal/external phloem parenchyma, xylem parenchyma, and internal/external pericycle, (Table II, Fig. 3). Fields of 1.2 mm x 0.1 mm were identified in each section which contain internal phloem, xylem, and external phloem strands. The total numbers of fields positive for fluorescence in each cell type was scored and the data collected from 80-100 fields treated with TGBp1 or CP antisera. The percentage of fields positive for immunofluorescence labeling showed GFP:TGBp1 and GFP:CP in most cells associated with the vasculature (Table II, Fig. 3) and in the pericycle. For controls, nontransgenic samples were treated with TGBp1 and CP antisera. While label appeared in some sections treated with each antiserum (Table II), the percentage values obtained from transgenic fields was significantly greater than the proportion of nontransgenic fields showing positive results for each cell type. Some samples were also treated with buffer and secondary antisera. None of these produce positive results indicating that any nonspecific labeling is likely to due the PVX antisera cross reacting with some host proteins (Table II). These data indicate that GFP:TGBp1 and GFP:CP exits the companion cells and moves laterally into sieve elements, phloem parenchyma, xylem parenchyma, and neighboring pericycle.

Table II. *Immunofluorescent labeling of LR white embedded cross sections derived from transgenic, non transgenic and infected N. benthamiana petioles*.

Each section two fields (1.2 mm x 0.1 mm) were scored for the presence of fluorescence in vascular tissue. Values represent the number of fields showing fluorescence in each cell type: Internal companion cells (ICC), Internal sieve element (ISE), Internal phloem parenchyma (IPP), External companion cells (ECC), External sieve element (ESE), External phloem parenchyma (EPP), Xylem parenchyma (XP), Internal pericycle (IPC), and External pericycle (EPC). Values in parentheses indicate the percentage of fields that were positive for fluorescence.

			Percentage of fields with fluorescent signals[a]								
Genotype	Sera	Fields	ICC	ISE	IPP	ECC	ESE	EPP	XP	IPC	EPC
Experiment-1											
*Nb*GFP:TGBp1-201	TGBp1	80	72(90%a)	61(76.3a)	56(70.0a)	76(95.0a)	75(93.7a)	66(82.5a)	80(100a)	69(86.3a)	12(15.0b)
*Nb*GFP:TGBp1-201	Buffer[b]	10	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0d)	0(0.0d)	0(0.0d)
N. benthamiana+PVX	TGBp1[c]	80	71(88.8a)	70(87.5a)	56(70.0a)	80(100a)	73(91.5a)	60(75.0a)	55(68.8b)	37(46.3b)	38(47.5a)
N. benthamiana	TGBp1	80	28(35.0b)	26(32.5b)	19(23.7b)	24(30.0b)	26(32.5b)	25(31.3b)	19(23.8c)	15(18.8c)	8(10.0c)
N. benthamiana	Buffer[d]	25	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0c)	0(0.0d)	0(0.0d)	0(0.0c)
Experiment-2											
*Nb*GFP:CP-207	CP	100	58(58.0a)	49(49.0a)	36(36.0a)	58(58.0a)	41(41.0a)	37(37.0a)	12(12.0b)	16(16.0b)	12(12.0b)
*Nb*GFP:CP-207	Buffer[b]	15	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)
N. benthamiana+PVX	CP[c]	100	50(50.0a)	47(47.0a)	45(45.0a)	50(50.0a)	43(43.0a)	35(35.0a)	40(40.0a)	41(41.0a)	34(34.0a)
N. benthamiana	CP	100	6(6.0b)	6(6.0b)	8(8.0b)	3(3.0b)	8(8.0b)	6(6.0b)	0(0.0c)	4(4.0c)	4(4.0c)
N. benthamiana	Buffer[d]	25	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0b)	0(0.0c)

[a] Statistical analysis was conducted down each column for each sera to demonstrate that TGBp1 or CP sera significantly labeled the transgenic plants and virus infected plants. Means followed by the same letter with in a column and an experiment are not significantly different at 5% probability. Label was minimal in nontransgenic tissue or samples treated with buffer.
[b] Samples are first treated with buffer in place of primary antisera and then treated with secondary antisera
[c] PVX-GUS infected samples are treated with PVX TGBp1 or CP antisera.
[d] *N. benthamiana* control is listed twice for statistical comparison.

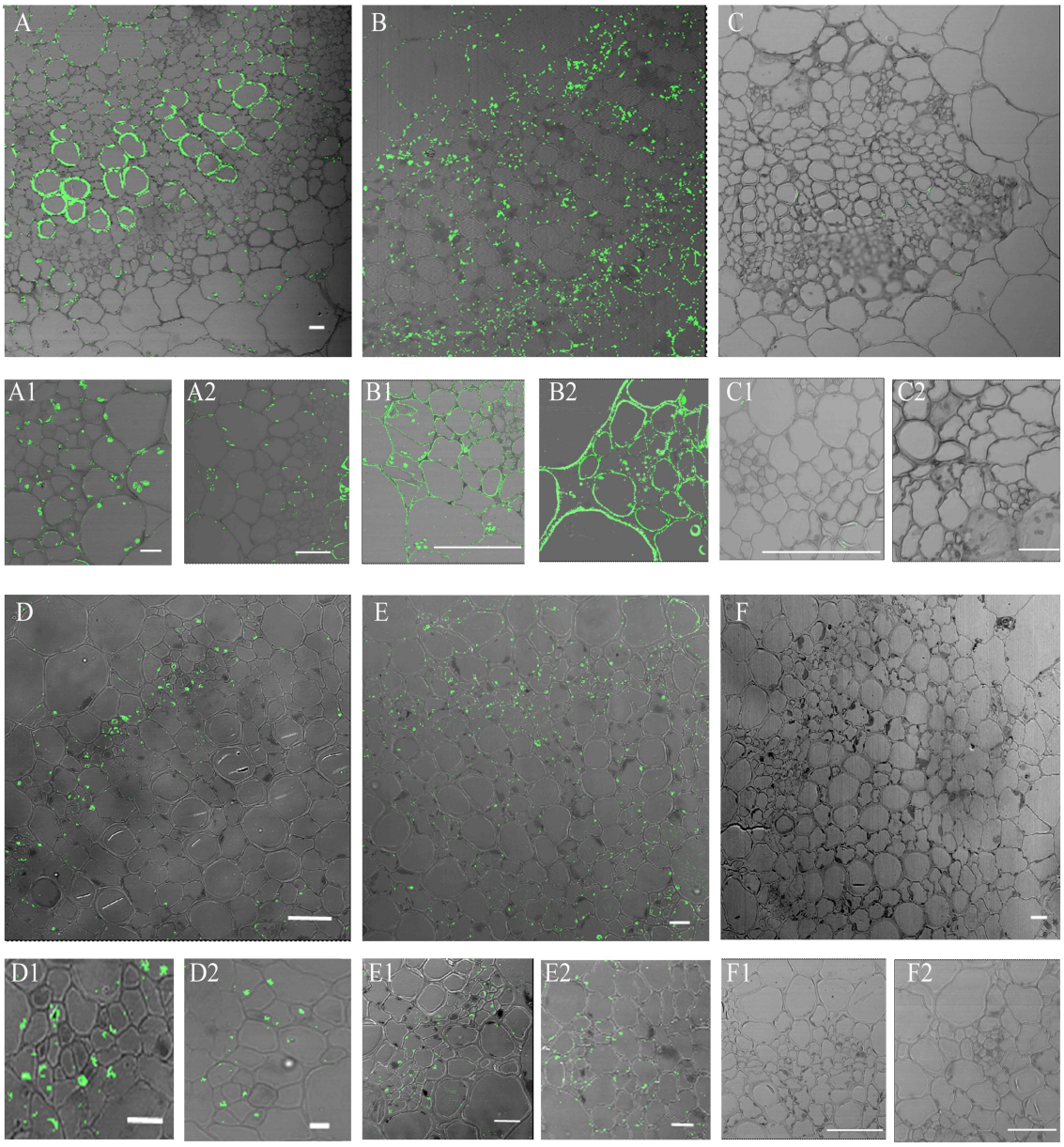


Figure 3. Confocal images of immunolabeled LR white embedded petiole cross sections. A, *Nb*GFP:TGBp1-201 labeled with anti-TGBp1 serum. A1 and A2 external and eternal phloem bundle regions. B, PVX-GUS infected *N. benthamiana* labeled with Anti-TGBp1 serum. B1 and B2, external and internal phloem bundle regions. C, *N. benthamiana* labeled with Anti-TGBp1 serum. C1 and C2, external and internal phloem bundle regions. D, *Nb*GFP:CP-207 labeled with anti-CP serum. D1 and D2 external and

eternal phloem bundle regions. E, PVX-GUS infected *N. benthamiana* labeled with Anti-TGBp1 serum. E1 and E2, external and internal phloem bundle regions. F, *N. benthamiana* labeled with Anti-TGBp1 serum. F1 and F2, external and internal phloem bundle regions. Bars represent 20μM.

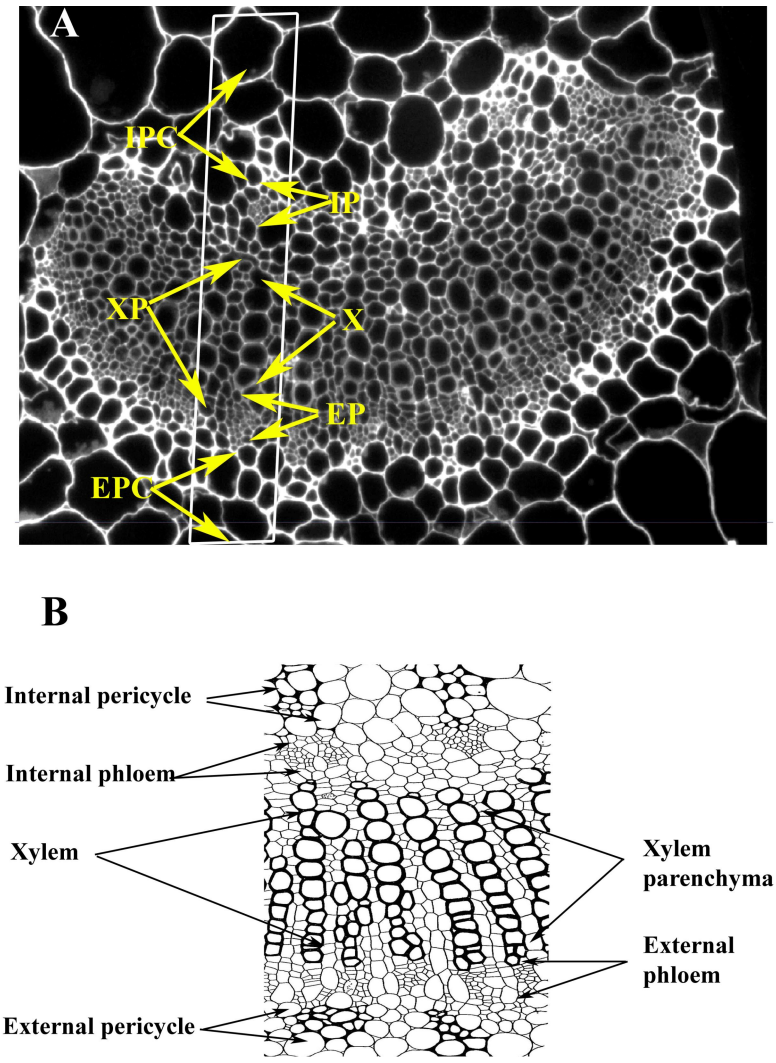


Figure 4. Images showing structure of a petiole cross section. A. Cross section of LR white embedded *Nb*GFP:TGBp1-201. The white box represents a field (1.2 mm x 0.1 mm) scored for the presence of fluorescence in vascular tissue. IP, Internal phloem; EP, external phloem; IPC, Internal pericycle; EPC, External pericycle; XP, Xylem parenchyma; X, Xylem. B, Detail drawing of portion of vascular arc of tobacco mid vein [from Avery (1933), Fig 39; Am J Bot 20(3):565-592]. The figure was reproduced with copyright permission of American Journal of Botany.

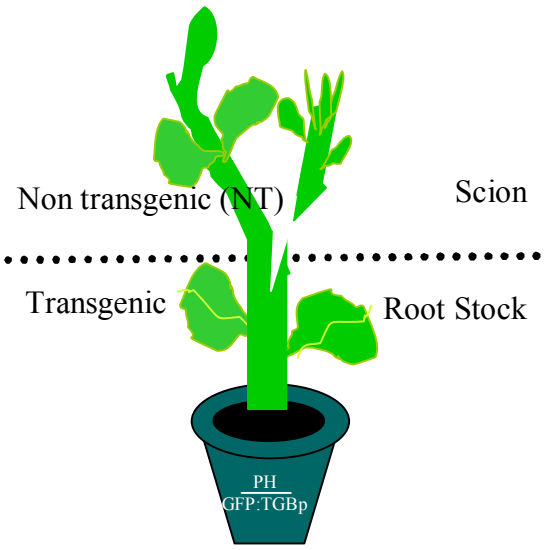
Transgenically Expressed Proteins Translocate Through Graft Union

Prior studies using phloem specific promoters to express the CMV movement protein (Itaya et al., 2002) demonstrated that proteins can be translocated through the phloem across a graft junction. Proteins entering the sieve element in the root stock are translocated by bulk flow across a graft union into sink leaves (Golecki et al., 1998; Golecki et al., 1999; Imlau et al., 1999; Aoki et al., 2005). In this study non transgenic scions were grafted onto *Nt*GFP:TGBp1-101, *Nt*GFP:TGBp2-102, *Nt*GFP:CP-117, or *Nt*GFP:GFP-105 rootstocks to determine protein movement through the graft union to scion (Table III). As a negative control, nontransgenic scions were grafted to *Nt*COI-107 root stocks. As positive controls, nontransgenic scions were grafted to either PVX-GUS infected *N. tabacum* or nontransgenic rootstocks treated with the phloem translocatable CF dye to verify that the grafts succeed in creating a continuum between the phloem strands.

Five plants were grafted for each transgenic line. Fluorescence was analyzed 28 days post grafting in the stem, approximately 4 cm above the graft union. When *Nt*GFPTGBp1-101, *Nt*GFPTGBp2-102, *Nt*GFPCP-107, *Nt*GFPGFP-105, or *Nt*GFP-108 were used as root stocks, fluorescence was seen in all nontransgenic scions (Table III). In scions grafted onto *Nt*COI-107 rootstock no fluorescent signal was detected in stem sections above the graft union. When PVX-GUS infected root stocks were used, a typical mosaic symptom of PVX was detected in the scion leaves approximately three weeks after grafting (Table III). CF dye was efficiently translocated through grafted plants within six hours after application of the dye at the bottom leaf of the root stock.

Table III. *Summary of grafting experiments*.

Scions established above the graft union were analyzed for translocation of GFP, PVX or CF signal from rootstock into scion through phloem. The total number of plants analyzed and the presence (+) or absence (-) of signal was indicated.

Graft type[a]	No. Plants	Scions & Root stocks	Result[b]
	5	NT / *Nt*GFPTGBp1-101	+ (5/5)
	5	NT / *Nt*GFPTGBp2-102	+ (5/5)
	5	NT / *Nt*GFPCP-107	+ (5/5)
	5	NT / *Nt*GFPGFP-105	+ (5/5)
	5	NT / *Nt*GFP-108	+ (5/5)
	5	NT / *Nt*COI-107	+ (0/5)
	5	NT / NT+PVX-GUS	+[c] (5/5)
	5	NT / NT+CF Dye	+[d] (5/5)

[a] Schematic drawing of the type of graft made using non transgenic scion and transgenic rootstocks or non transgenic rootstocks.
[b] Stem segment up to 4 cm above the graft union was analyzed for GFP.
[c] PVX symptom detected in the scion 3 weeks after grafting on infected root stock.
[d] 5(6) carboxyfluorescein diacetate (CF) dye detected in scion 6 h after application on rootstock.

DISCUSSION

In this study transgenically expressed GFP:TGBp1, GFP:TGBp2, GFP:CP, GFP:GFP, and GFP are restricted to the phloem in all classes of leaf veins irrespective of the age of the leaf (Fig. 1A, D, G, J). Using hand sections of live tissues and immunofluorescence labeling of embedded *Nb*GFP:TGBp1-201 and *Nb*GFP:CP-207 petioles, fluorescence was detected in the xylem parenchyma and pericycle (Tables I and II). In the stems of *Nb*GFP:TGBp1-201 transgenic plants, fluorescence was also detected in the pith. While GFP:GFP and GFP were phloem restricted, these data indicate that the PVX TGBp1 and CP are both able to direct GFP out of the phloem companion cells into neighboring vascular tissues. Only GFP:TGBp1 was seen in the pith of the stem, indicating that the PVX TGBp1 protein has the ability to overcome barriers to phloem exit in the stem and spread further beyond the vasculature than the other transgenically expressed proteins. The PVX TGBp1 and CP have been reported to interact with plasmodesmata (Oparka et al., 1996; Howard et al., 2004). Only TGBp1 has the ability to gate open plasmodesmata to allow movement of macromolecules between cells (Howard et al., 2004). If the plasmodesmata provide a barrier to diffusion of proteins out of the phloem, then TGBp1, and possibly CP likely acts to gate plasmodesmata in the petiole for its own movement out of the companion cells into surrounding tissues.

While we were unable to detect GFP:CP in these tissues using live sections, immunofluorescence labeling served to amplify the signal to detect low levels of protein accumulating outside the companion cells. If we compare samples treated with TGBp1 and CP antisera, the percentage of fields showing fluorescence due to GFP:TGBp1 in the xylem parenchyma and pericycle are at least 3 fold greater than for GFP:CP indicating

that less GFP:CP proteins accumulate in these tissues. TGBp1 also differs from CP in its ability to interact with plasmodesmata in the stem to promote its own movement into nonvascular cells. Thus while both PVX proteins appear to move from cell-to-cell, they differ in their mobility or accumulation patterns.

Recent studies of destination-selective long-distance trafficking of phloem proteins (Aoki et al., 2005) suggested that protein accumulation outside of the vasculature is highly regulated by cellular degradation machinery. Cellular chaperones located in the phloem guide proteins from the cells where they are first expressed to specific destinations. Proteins moving out of the phloem through the plasmodesmata and into neighboring cells can be quickly degraded in nontarget tissues. Essential proteins accumulate in target tissues while other proteins are degraded. Accepting this model, it is reasonable to conclude that the transgenically expressed proteins exited the leaf veins, but were degraded in the cells surrounding the veins. This would have resulted in undetectable or sporadic detection of proteins in cells neighboring the veins. Thus, GFP:TGBp1, GFP:TGBp2, GFP:CP, GFP:GFP, and GFP may have been similarly affected by the cells degradation machinery in the leaves. The sporadic detection of PVX proteins in leaf cells neighboring the veins and evidence of fluorescence in the petiole and stem in cells surrounding the vasculature can best be explained by a selective mechanism which may target certain proteins for degradation while allowing others to accumulate. Further experiments will be conducted using protease inhibitors to determine if protein degradation can be blocked in the leaves and improve our ability to detect fluorescence accumulation in tissues surrounding the veins. Alternatively, evidence of GFP:TGBp1 and GFP:CP in the petiole and stem suggests that: i)

GFP:TGBp1 and GFP:CP may be able to evade the degradation machinery; ii) a specific degradation mechanism act on GFP:TGBp2 which does not affect GFP:TGBp1 and GFP:CP; and iii) TGBp1 and CP have the ability to overcome specific barriers to phloem exit in the petiole and stem. Perhaps TGBp1 and CP interact with unique features of the plasmodesmata in the petiole and stem to promote their own cell-to-cell movement.

Transgenic plants were inoculated with PVX-GUS and GFP:TGBp1 and GFP:CP, but not GFP:TGBp2, GFP:GFP, or GFP were seen to unload in leaves. These data suggest that the virus interacts with TGBp1 or CP, but not TGBp2 or GFP carrying the fusions into tissues surrounding the veins. Thus PVX-GUS does not gate open plasmodesmata to allow nonspecific movement of proteins into surrounding tissues but specifically interacts with GFP:TGBp1 and GFP:CP. Prior studies showed that the potexvirus TGBp1 and CP are translocatable through the phloem by grafting transgenic rootstocks and nontransgenic scions (Lough et al., 2001; Santa Cruz et al., 1998). Studies exploring vascular transport of PVX failed to detect virions in the phloem and led to a model in which PVX moves through the vasculature as a ribonucleoprotein complex that includes the viral CP. Further support for this model was presented in Lough et al., (2001) where they demonstrated that mutant viral RNAs were translocated across a graft junction when the rootstock transgenically expressed the entire TGB. Evidence that TGBp1 is phloem mobile and that the entire TGB is not important for virus exit into the leaves led these researchers to suggest that there is a ribonucleoprotein complex containing viral RNA, TGBp1, and CP which moves through the phloem and exits in young leaves.

The data we present in this paper support the Santa Cruz and Lough models because GFP:TGBp1 and GFP:CP exit the veins in tobacco leaves only in the presence of PVX. These data suggest that there is a specific interaction between the virus and these two proteins for phloem exit. Since GFP:TGBp2 does not exit the veins along with the virus, these data support Lough's contention that TGBp2 is dispensable for virus unloading from the phloem. In this study we expressed the GFP fusions using companion cell specific promoters while our colleagues reported using transgenic plants constitutively expressing the viral proteins. Thus we have the unique ability to study phloem exit without requiring the use of grafting experiments.

Roberts et al., (1997) reported vein class III functions for the exit of macromolecules from the phloem into surrounding nonvascular tissues (i.e. mesophyll and epidermal) while vein class IV and V function only form phloem loading in source leaves and are not mature enough to function in sink leaves. This hypothesis was supported by studies showing that certain RNA viruses (PVX, BSMV, and CPMV), as well as CF dye exit the phloem in leaves through vein class III but not vein class IV or V (Roberts et al., 1997; Haupt et al., 2001; Silva et al., 2002). Research presented in Table I and Fig 2 shows that the transgenically expressed GFP:TGBp1 and GFP:CP proteins unload from vein class III-V in PVX infected plants. These data indicate that class IV and V veins are functional for protein exit from the phloem.

Fluorescence detected in tissues surrounding class IV and V veins following PVX infection indicates that either the virus is creating an opportunity for the proteins to exit the vasculature, or is enhancing protein accumulation in the surrounding tissues to detectable levels. If the proteins are degraded in the mesophyll cells, according to the

destination selective model, perhaps the presence of the virus protects them from degradation and thereby enhances their accumulation. The Lough and Santa Cruz models suggest that TGBp1 and CP form a complex with viral RNA which exits the phloem. Perhaps an RNP complex is more stable and/or resistant to protease degradation and therefore we were able to detect fluorescence in the nonvascular cells in the leaves.

LITERATURES CITED:

Aoki K, Kragler F, Xoconostle-Cazares B, Lucas WJ (2002) A subclass of plant heat shock cognate 70 chaperones carries a motif that facilitates trafficking through plasmodesmata. Proc Natl Acad Sci USA **99:** 16342-16347

Aoki K, Suzui N, Fujimaki S, Dohmae N, Yonekura-Sakakibara K, Fujiwara T, Hayashi H, Yamaya T, Sakakibara H (2005) Destination-selective long-distance movement of phloem proteins. Plant Cell **17:** 1801-1814

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

Blackman LM, Boevink P, Santa Cruz S, Palukaitis P, Oparka KJ (1998) The movement protein of *Cucumber mosaic virus* traffics into sieve elements in minor veins of *Nicotiana clevelandii*. Plant Cell **10:** 525-538

Chapman S, Kavanagh T, Baulcombe D (1992) *Potato virus X* as a vector for gene expression in plants. The Plant Journal **2:** 549-557

Cheng N-H, Su C-L, Carter SA, Nelson RS (2000) Vascular invasion routes and systemic accumulation patterns of *Tobacco mosaic virus* in *Nicotiana benthamiana*. Plant J **23:** 349-362

Driskel BA, Doss P, Littlefield LJ, Walker NR, Verchot LJ (2004) *Soilborne Wheat mosaic virus* movement protein and RNA and *Wheat spindle streak mosaic virus* coat protein accumulate inside resting spores of their vector, *Polymyxa graminis*. Mol Plant Microbe Interact **17:** 739-748

Esau K (1965) Plant anatomy, Ed 2nd. John Wiley & Sons, Inc., New York

Golecki B, Schulz A, Carstens-Behrens U, Kollmann R (1998) Evidence for graft transmission of structural phloem proteins or their precursors in heterografts of Cucurbitaceae. Planta **206:** 630-640

Golecki B, Schulz A, Thompson G (1999) Translocation of structural P proteins in the phloem. Plant Cell **11:** 127-140

Haupt S, Duncan GH, Holzberg S, Oparka KJ (2001) Evidence for symplastic phloem unloading in sink leaves of barley. Plant Physiol **125:** 209-218

Howard AR, Heppler ML, Ju HJ, Krishnamurthy K, Payton ME, Verchot-Lubicz J (2004) *Potato virus X* TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves. Virology **328:** 185-197

Imlau A, Truernit E, 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, Ma F, Qi Y, Matsuda Y, Zhu Y, Liang G, Ding B (2002) Plasmodesma-mediated selective protein traffic between "symplasmically isolated" cells probed by a viral movement protein. Plant Cell **14:** 2071-2083

Ju HJ, Brown JE, Ye CM, Verchot-Lubicz J (2007) Mutations in the central domain of *Potato virus X* TGBp2 eliminate granular vesicles and virus cell-to-cell trafficking. J Virol **81:** 1899-1911

Ju HJ, Samuels TD, Wang YS, Blancaflor E, Payton M, Mitra R, Krishnamurthy K, Nelson RS, Verchot-Lubicz J (2005) The *Potato virus X* TGBp2 movement

protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol **138:** 1877-1895

Karpova OV, Zayakina OV, Arkhipenko MV, Sheval EV, Kiselyova OI, Poljakov VY, Yaminsky IV, Rodionova NP, Atabekov JG (2006) *Potato virus X* RNA-mediated assembly of single-tailed ternary 'coat protein-RNA-movement protein' complexes. J Gen Virol **87:** 2731-2740

Kathryn MW, Oparka KJ (1996) The fluorescent probe HPTS as a phloem-mobile, symplastic tracer: an evaluation using confocal laser scanning microscopy. J Exp Bot **47:** 439-445

Krishnamurthy K, Heppler M, Mitra R, Blancaflor E, Payton M, Nelson RS, 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

Lough TJ, Emerson SJ, Lucas WJ, Forster RL (2001) Trans-complementation of long-distance movement of *White clover mosaic virus* triple gene block (TGB) mutants: phloem-associated movement of TGBp1. Virology **288:** 18-28

Lough TJ, Lee RH, Emerson SJ, Forster RL, Lucas WJ (2006) Functional analysis of the 5' untranslated region of potexvirus RNA reveals a role in viral replication and cell-to-cell movement. Virology **351:** 455-465

Lough TJ, Netzler NA, Emerson SJ, Sutherland P, Carr F, Beck DL, Lucas WJ, Forster RL (2000) Cell-to-cell movement of potexviruses: evidence for a ribonucleic protein complex involving the coat protein and first triple gene block protein. Mol Plant Microbe Interact **13:** 13:962–974

Lucas WJ, Wolf S (1999) Connections between virus movement, macromolecular signaling and assimilate allocation. Curr Opin Plant Biol **2:** 192-197

Nelson RS, van Bel AJE (1998) The mystery of virus trafficking into, through, and out of vascular tissue. Prog Bot **59:** 476-533

Oparka KJ, Boevink P, Santa Cruz S (1996) Studying the movement of plant viruses using green fluorescent protein. Trends in Plant Science **1:** 412-418

Oparka KJ, Santa Cruz S (2000) The great escape: Phloem transport and unloading of macromolecules. Annu Rev Plant Physiol Plant Mol Biol **51:** 323-347

Reichel C, Beachy RN (2000) Degradation of *Tobacco mosaic virus* movement protein by the 26S proteasome. J. Virol. **74:** 3330-3337

Roberts AG, Santa Cruz S, Roberts IM, Prior D, Turgeon R, Oparka KJ (1997) Phloem unloading in sink Leaves of *Nicotiana benthamiana*: comparison of a fluorescent solute with a fluorescent virus. Plant Cell **9:** 1381-1396

Silva MS, Wellink J, Goldbach RW, van Lent JW (2002) Phloem loading and unloading of *Cowpea mosaic virus* in *Vigna unguiculata*. J Gen Virol **83:** 1493-1504

Tamai A, 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. Mol Plant Microbe Interact **14:** 1158-1167

Trip P, Gorham PR (1968) Bidirectional translocation of sugars in sieve tubes of squash plants. Plant Physiol **43:** 877-889

Verchot LJ (2005) A new cell-to-cell transport model for Potexviruses. Mol Plant Microbe Interact **18:** 283-290

Voinnet O, Vain P, Angell S, Baulcombe DC (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell **95:** 177-187

Wu X, Weigel D, Wigge PA (2002) Signaling in plants by intercellular RNA and protein movement. Genes Dev. **16:** 151-158

Xoconostle-Cazares B, Xiang Y, Ruiz-Medrano R, Wang HL, Monzer J, Yoo BC, McFarland KC, Franceschi VR, Lucas WJ (1999) Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. Science **283:** 94-98

CHAPTER IV

SUBCELLULAR DISTRIBUTION OF GFP:TGBp1 AND GFP:CP IN THE VASCULATURE OF TRANSGENIC TOBACCO PLANTS

ABSTRACT

Experiments were conducted using the transgenic plants generated in Chapter II to determine the subcellular distribution of PVX TGBp1, TGBp2, and CP in the vasculature. Leaf veins from *Nb*GFP:TGBp1-201, *Nb*GFP:CP-207, *Nb*GFP:GFP-201 and *Nb*GFP-205 from live plants were viewed using epifluorescent and confocal microscopy. In major and minor veins, GFP:TGBp1 was identified in elongated fluorescent bodies. GFP:CP fluorescence occurred in the cytoplasm and round mobile fluorescent bodies inside sieve elements of major and minor veins. In both *Nb*GFP:GFP-201 and *Nb*GFP-205, fluorescent signals often detected in the nucleus and in cytosol of companion cells. The signals from *Nb*GFP:GFP-201 was very strong. Immunogold labeling and transmission electron microscopy were used to characterize the subcellular distribution of GFP:TGBp1 and GFP:CP in sieve elements and companion cells. Gold particles in *Nb*GFP:TGBp1 accumulated in the chloroplast, plastid, vacuole and cytosol of companion cells, and associated with plastids and P-proteins in sieve elements. In *Nb*GFP:CP-207 gold particles accumulated in plastid and cytosol of companion cells and plastids and cytoplasm of sieve elements. *Nb*GFP:TGBp1 transgenic plant showed an increased number of starch bodies in xylem parenchyma, pericycles and endodermal

cells and fewer tracheary elements were seen in cross sections. In *Nb*GFP:CP-207 there were more tracheary elements seen in cross sections.

INTRODUCTION

Plant viruses utilize movement proteins to spread cell to cell through plasmodesmata and over a long distance through phloem (Carrington et al., 1996; Gilbertson and Lucas, 1996; Mezitt and Lucas, 1996; Santa Cruz et al., 1996; Carrington et al., 1998; Lazarowitz and Beachy, 1999; Lucas and Wolf, 1999; Itaya et al., 2002). Plasmodesmata are membrane-lined channels which function as transport pathways between plant cells (Ding et al., 1992). The phloem is nutrient-conducting tissue of vascular plants, consisting mainly of companion cells, sieve elements and phloem parenchyma (Esau, 1965, , 1973; Evert, 1982). The companion cells and sieve elements originated from the same precursor cells and work in coordination to facilitate vascular transport (Esau, 1965). Companion cells typically have larger numbers of ribosome and mitochondria which make them more metabolically active than a typical plant cell. Young sieve cells have the same organelles as mesophyll cells and other cells, but as sieve cells undergo significant modifications including highly selective autolysis of several organelles and a process of selective degradation resulting in the mature enucleated sieve elements (Esau, 1965; Sjolund, 1997; Oparka and Turgeon, 1999). The entire sieve element protoplast, the central vacuole, cytoplasmic ribosome, Golgi bodies, and nucleus are selectively degraded and eventually eliminated (Oparka and Turgeon, 1999). The mature enucleated sieve elements retain a highly degenerated cytoplasm, but they can remain viable and functional for decades (Parthasarathy and Larry, 1976; Oparka and Turgeon, 1999). Mature sieve elements retain certain organelles including filamentous phloem proteins (P-proteins), stacked cisternal aggregates of ER

concentrated at the lateral walls, plasma membrane, modified mitochondria; plastids and few vacuoles (Cronshaw and Esau, 1967).

In many studies, GFP has been fused to viral movement proteins to visually characterize the subcellular distribution of these proteins in live cells (Solovyev et al., 2000; Yang et al., 2000; Lawrence and Jackson, 2001; Rojas et al., 2001; Krishnamurthy et al., 2002; Krishnamurthy et al., 2003; Mitra et al., 2003; Howard et al., 2004; Ju et al., 2005; Ju et al., 2007). The expression, function and the subcellular targeting of viral movement proteins can be studied in following ways: 1) transfecting protoplasts with virus or plasmids expressing viral movement proteins to study protein subcellular targeting and expression (Reichel and Beachy, 2000; Rojas et al., 2001; Toth et al., 2001; Ju et al., 2005; Ju et al., 2007). Protoplasts isolated from mesophyll or derived from cultured cells have been exploited in several plant species for rapid gene functional analysis and biochemical manipulations of movement proteins (Karpova et al., 1997; Rojas et al., 2001; Ju et al., 2005; Ju et al., 2007). 2) Transient expression via particle bombardment of detached leaves is also widely used to study protein expression in the absence of virus infection in intact leaves (Itaya et al., 1997; Crawford and Zambryski, 2001; Krishnamurthy et al., 2002; Krishnamurthy et al., 2003; Mitra et al., 2003). 3) Infecting host plants or protoplast directly with virus carrying GFP or β-glucuronidase (GUS) to understand how plant viruses move and accumulate in their hosts (Krishnamurthy et al., 2002; Krishnamurthy et al., 2003; Mitra et al., 2003; Howard et al., 2004; Ju et al., 2005; Ju et al., 2007). 4) Using transgenic plants (Citovsky et al., 1993; Itaya et al., 2002; Ajjikuttira et al., 2005) expressing viral movement proteins. Transgenic plants expressing movement proteins have been valuable tools for studying

the subcellular accumulation patterns as well as protein function in the absence of virus infection.

Transgenic expression of virus movement proteins can have certain effects on plant development. In transgenic *N. tabacum* expressing TMV movement protein using the phloem specific *rolC* promoter reported to alters carbohydrate metabolism in source leaves and dry matter partitioning between shoot, root and tubers (Almon et al., 1997). In this report, starch and sucrose accumulation in source leaves was significantly higher in transgenic than in nontransgenic plants indicating that the stress induced by movement proteins in transgenic plant alters allocation of dry matter in favour of particular organ. Constitutive expression of *Tomato spotted wilt virus* (TSWV) NS_M viral movement protein using the CaMV 35S promoter in *N. tabacum* was also found to induce severe, TSWV like symptoms, with highly reduced root and shoot development (Rinne et al., 2005). In this study the authors observed reduced leaf expansion and internode elongation in plants expressing NS_M. Interestingly, during sink-source transition they observed an accumulation of excessive amounts of starch in the mesophyll due to obstruction of mesophyll plasmodesmata as a result of deposition of 1,3-beta-D-glucan or callose triggered by expression of the NS_M proteins (Rinne et al., 2005).

Potato virus X (PVX) has four movement proteins, the first three encoded from a genetic module of three overlapping open reading frames (ORFs), termed the triple gene block and the fourth one is CP. These triple gene block proteins are named TGBp1, TGBp2, and TGBp3 (Huisman et al., 1988). The PVX TGBp1 protein is a suppressor of gene silencing, an RNA helicase, and gates plasmodesmata (Howard et al., 2004). TGBp2 and TGBp3 are ER associate PVX movement proteins (Tamai and Meshi, 2001;

Krishnamurthy et al., 2003; Ju et al., 2005; Ju et al., 2007). The PVX coat protein is required for encapsidation and virus movement (Spillane et al., 1997). PVX TGBp1 together with viral RNA and CP form a RNP complex and co-transport along the phloem sieve element to spread systemically (Lough et al., 2000; Morozov and Solovyev, 2003). Earlier work with immunogold labeling of thin section of PVX infected tissue (Davies et al., 1993) revealed that TGBp1 is associated with cytoplasmic inclusion bodies. Recently, it has been shown that TGBp2 and TGBp3 are both associated with ER, and TGBp3 is also associated with ER derived vesicles in protoplast and PVX-GFP infected leaves (Mitra et al., 2003; Ju et al., 2005; Ju et al., 2007). To our knowledge there is no report on how PVX movement proteins behave in the vasculature.

In this study, the green fluorescent protein (GFP) gene was fused to the PVX TGBp1 and CP genes and transgenically expressed in *N. tabacum* and *N. benthamiana* to study protein subcellular targeting in vasculature of transgenic plants. We used immunogold for labeling ultrathin longitudinal sections from petiole to detect distribution of TGBp1 and CP proteins using transmission electron microscopy. We also present evidence that TGBp1 induces accumulation of starch bodies in petioles. Lastly, we present evidence that the PVX TGBp1 and CP proteins alter xylem development in transgenic plants.

MATERIALS AND METHODS

Fixation and Embedding of Petioles

Petiole cross sections from nontransgenic *N. benthamiana* and the transformants *Nb*GFP:TGBp1-201, *Nb*GFP:TGBp2-204, *Nb*GFP:CP-207, *Nb*GFP:GFP-201, *Nb*GFP-205 and *Nb*COI-203 plants were fixed for 2 h at room temperature under vacuum in a 10 ml solution containing 0.5% (v/v) glutaraldehyde (v/v), 4.0% paraformaldehyde (v/v), and 34.2 g L^{-1} sucrose in 50 mM sodium cacodylate buffer (v/v) (pH 7.2) (Verchot et al., 2001; Driskel et al., 2004). The samples were embedded in LR White resin (Ted Pella, Redding, CA) following procedures described previously (Verchot et al., 2001; Driskel et al., 2004). Samples were dehydrated through ethanol series, (30%, 50%, 70%, 90%, 95%, 100%, 100%, 100%) for 20 minutes per step. Then samples were infiltrated overnight using 2:1 (v/v) ethanol/ LR white mixture. Infiltration was repeated first with 1:1 ethanol/ LR white mixture and then 100% LR white, each overnight. Finally samples were placed at the bottom of the capsules and capsules were filled with 100% LR white and placed in oven under vacuum at 60 ^{o}C overnight for polymerization of the resin. Blocks were stored at room temperature until used.

Immunogold Labeling of LR-White Embedded Ultrathin Section

Ultrathin sections (60 nm) of LR-White embedded petioles of *Nb*GFP:TGBp1-201, *Nb*GFP:CP-207, *Nb*COI-205 and nontransgenic *N. benthamiana* were cut with a diamond knife on a Sorvall MT 6000 ultramicrotome and mounted on formvar-carbon coated nickel grids (Electron Microscopy Science, Hatfield, PA) (Ju et al., 2005). Immunogold labeling procedures were done following the procedures described

previously (Ju et al., 2005). Immunogold labeling was conducted using goat derived polyclonal TGBp1 antisera to detect TGBp1 (prepared by Chang-Ming Ye in our laboratory) and rabbit derived PVX antisera (Agdia, Inc.) to detect PVX CP. Grids were incubated in blocking solution consisting of PBS, pH 7.5 (130 mM NaCl, 7.0 mM Na_2HPO_4, 3.0 mM NaH_2PO_4) plus 2% bovine serum albumin (BSA; w/v) for 30 min, and then incubated with 2% normal sheep serum (Rockland Immunochemicals, Inc. Gilbertsville, PA) in PBS plus 2% BSA for 15 min. Then *Nb*GFP:TGBp1 samples were incubated with anti-TGBp1 sera diluted 1:126 in PBS plus 2% BSA (w/v) and GFP:CP samples with anti-PVX sera (Agdia) diluted 1:100 in PBS plus 2% BSA for 2 h. Sections from *N. benthamiana* or *Nb*COI were treated in same way either with anti-TGBp1 or anti-PVX sera. For non-specific binding control of secondary antisera, *Nb*GFP:TGBp1, *Nb*GFP:CP, *Nb*COI samples and a nontransgenic control were treated with buffer containing no primary antisera for 2 h. All grids were then washed five times for 5 min with PBS and then with PBS plus 2% fish gelatin (v/v) for 15 min. *Nb*GFP:TGBp1 and control grids were incubated for 1 h with 20 nm gold-conjugated goat antisera (EY Labs, San Mateo, CA) of 1:10 dilution (v/v) in PBS plus 2% fish gelatin. Similarly *Nb*GFP:CP and control grids were incubated for 1 h in gold-conjugated rabbit (EY Labs) antisera of 1:10 dilution (v/v) in PBS plus 2% fish gelatin). All grids were washed three times for 5 min with distilled, deionized water, and stained with a solution of 2.5% uranyl acetate and 70% methanol (v/v) for 30 min, and then with a solution of 2% Reynold's lead citrate, pH 12.0 (in distilled, deionized water) for 20 min. Samples were washed with lukewarm distilled, deionized water three times for 5 min and then dried.

Toluidine Blue and IKI Staining of Petiole Cross Sections

Three live petiole cross sections from the fourth leave of ten day old *Nb*GFP:TGBp1-201, *Nb*GFP:TGBp2-204, *Nb*GFP:CP-207, *Nb*GFP:GFP-201, *Nb*GFP-205 and *Nb*COI-203 plants were prepared, placed on slides, stained with 0.1% Toluidine blue for one minute, excess stain was blotted with filter paper and tracheary elements were counted using bright field of epifluorescence microscopy. Five plants were used to count the numbers of tracheary elements inside the vascular arc of petiole cross sections.

A 1 % iodine potassium iodide (IKI) solution was prepared first by dissolving potassium iodide in 70% ethanol and then equal amount of iodine (w/w) was added and allowed to completely dissolve (Cutter, 1969; Berlyn and Miksche, 1976). IKI stains starch blue-black to orange depending on the type of starch present (Cutter, 1969; Berlyn and Miksche, 1976). Thick sections (500 nm) were cut with a glass knife on a Sorvall MT 6000 ultramicrotome and mounted on slides (Fisher Biotechnology, Pittsburgh, PA). Ten sections were mounted on the slide and flattened by heating on a hot plate at about 50^0C. Slides were flooded with IKI, a coverslip was applied, and then incubated for one minute. The excess IKI was removed by blotting one edge of the coverslip with a Kim wipe while adding water to the opposite edge (Cutter, 1969). IKI staining was then examined using bright field microscopy.

Bright Field, Confocal, and Transmission Electron Microscopy

A Nikon E600 microscope was used for bright field imaging. Images were captured using the Optronics Magnafire camera (Intelligent Imaging Innovations, Inc.). The Leica TCS SP2 system is attached to a Leica DMRE microscope (Leica

Microsystems, Bannockburn, IL). For GFP expression, 488 nm excitation wavelength produced by Krypton/Argon lasers were used. A 358-nm excitation wavelength was used to view 4',6'-diamidino-2-phenylindole hydrochloride (DAPI), and a 558-nm excitation wavelength was used to examine FM® 4-64 dye. Electron microscopic analysis of samples was carried out using a JEOL JEM-2100 with EDAX Genesis 2000 EDS system. All images were processed using Adobe Photoshop CS version 8.0 software (Adobe Systems, San Jose, CA).

Statistical Analysis

All quantitative data collected in this study were processed either with Excel or SAS 9.1 software package. The number of gold particles observed in the electron micrograph were counted in 70 fields (each with an area of 4 μm^2) and subjected to statistical analysis. ANOVA procedures with PC SAS version 9.1 (SAS Institute, Cary, NC) and PROC GLM (Ju et al., 2005) were used in Table I to compare: 1) subcellular localization of GFP:TGBp1 within companion cells or sieve elements treated with TGBp1 antisera ; 2) subcellular localization of GFP:CP in companion cells or sieve elements treated with CP antisera; 3) TGBp1 antisera treated *Nb*GFP:TGBp1-201 and *N. benthamiana* with buffer treated *Nb*GFP:TGBp1-201; 4) CP sera treated *Nb*GFP:CP-207 and *Nb*COI-203 with buffer treated *Nb*GFP:CP-207.

ANOVA procedures with PC SAS version 9.1 (SAS Institute) and PROC GLM were used to compare the numbers of tracheary elements seen in petiole cross sections of *Nb*GFP:TGBp1-201, *Nb*GFP:TGBp2-204, *Nb*GFP:CP-207, *Nb*GFP:GFP-201, *Nb*GFP-205 and *Nb*COI-203 plants.

For all above analysis when ANOVA was significant, pairwise comparisons of means were made with a PDIFF option in an LSMEANS statement. A significant level of 0.01 was used for all comparisons.

RESULTS

TGBp1 and CP Behave Differently in Phloem

Leaf sections from *Nt*GFP:TGBp1-101, *Nt*GFP:CP-117, *Nt*GFP:GFP-105, *Nt*GFP-108, *Nb*GFP:TGBp1-201, *Nb*GFP:CP-207, *Nb*GFP:GFP-201 and *Nb*GFP-205 T2 lines were viewed using epifluorescence and confocal microscopy to compare the subcellular accumulation patterns of the fusion proteins.

In *Nt*GFP:TGBp1-101 and *Nb*GFP:TGBp1-201, leaf fluorescence was predominantly seen in elongated bodies that were between 30-120 μm in companion cells. The pattern of fluorescence was the same in all vein classes from I-V (Fig 1. A and B). Some elongated bodies were mobile in the sieve elements of young leaves but not in mature fully expanded leaves. It is possible that these mobile bodies are crystalloid filamentous phloem proteins (also called P-proteins) (Knoblauch and van Bel, 1998).

In *Nt*GFP:CP-117 and *Nb*GFP:CP-207 fluorescence was seen in cytoplasm in companion cells and in mobile spheres in sieve elements. The pattern of fluorescence was the same in all vein classes from I-V (Fig.1C and D). Unlike *Nb*GFP:TGBp1-201 plants, the mobile spheres in *Nb*GFP:CP-207 transgenic plants seemed unaffected by the age of plant. The dimensions of the mobile spheres in *Nt*GFP:CP-117 and *Nb*GFP:CP-207 veins seemed to be different from mobile elongated bodies in *Nt*GFP:TGBp1-101 and *Nb*GFP:TGBp1-201 veins suggesting that the PVX TGBp1 and CP target to different mobile structures in sieve elements.

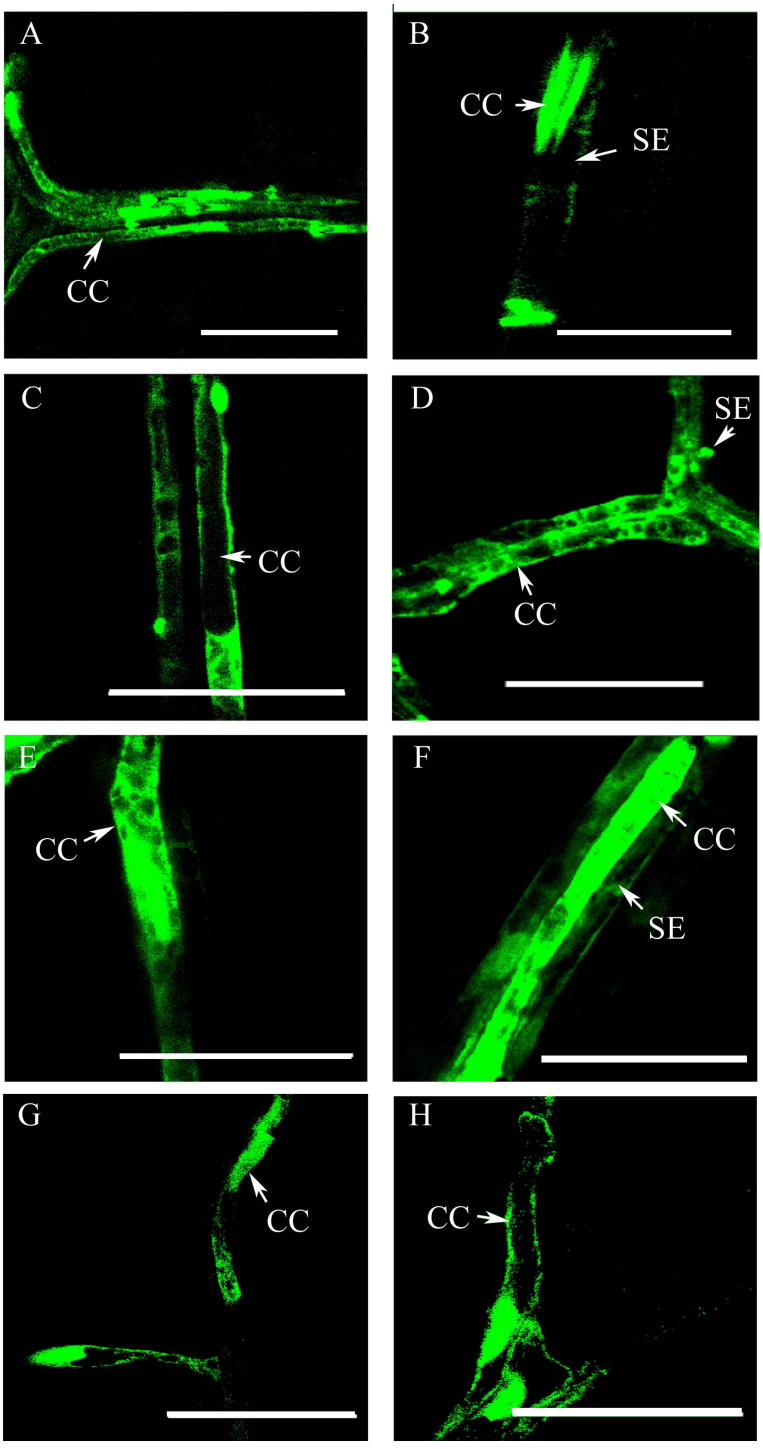


Figure 1. Confocal images of transgenic leaves expressing GFP fusion proteins. Companion cells (CC) and sieve element (SE) are identified in each panel. A and B,

Characteristic elongated fluorescent bodies can be seen in veins of a *Nb*GFP;TGBp1-201 leaf. C and D, Mobile spheres are seen in sieve elements but not in companion cells of *Nb*GFP:CP-207 leaf veins. E and F, Fluorescence is in the cytoplasm and nucleus in *Nb*GFP:GFP-201 leaf veins. G and H, Fluorescence is in the cytoplasm and nucleus of *Nb*GFP-205 and *Nb*GFP-203 leaf veins, respectively. Bars represent 50μM.

*Nt*GFP:GFP-105, *Nt*GFP-CP-207, *Nb*GFP:GFP-201, and *Nb*GFP-205 showed fluorescence in the nucleus, cytosol in companion cells in all vein classes (I-V) (Fig. 1E-H). Fluorescence due to GFP:GFP was stronger than GFP alone. Fluorescence was also seen in the sieve elements as expected (Fig. 1H).

Since companion cells contain nuclei, mitochondria, chloroplasts, vacuoles, plastids, cell membranes and cell walls, and mature sieve elements also contain organelles including P-proteins, stacked cisternal aggregates of ER, plasma membrane, modified mitochondria, plastids and few vacuoles it is likely that proteins expressed in companion cells or that enter sieve elements might target those cytoplasmic components or organelles. Our observations in leaf sections of *Nb*GFP:TGBp1-201, *Nb*GFP:CP-207, *Nb*GFP:GFP-201 and *Nb*GFP-205 T2 lines (Fig. 1) indicate that the PVX TGBp1 and CP target specific subcellular locations within companion cells and sieve elements.

Immunogold Labeling Shows GFP:TGBp1 and GFP:CP Localize Differently in Phloem

We hypothesized that the elongated bodies inside companion cells of *Nb*GFP:TGBp1-101 and *Nb*GFP:TGBp1-201 are vacuoles, plastids, or starch bodies (Fisher, 1991). We also hypothesized that the moving spheres in sieve elements of *Nt*GFP:CP-117 and *Nb*GFP:CP-207 transgenic plants are plastids. Immunogold labeling and electron microscopy were used to study the subcellular localization of GFP:TGBp1 and GFP:CP in companion cells and sieve elements.

Figure 2 shows a low magnification image of sieve elements and companion cells with the important structures highlighted. A more detailed analysis at higher

magnification was conducted for immunolabelling studies of *Nb*GFP:TGBp1-201 and *Nb*GFP:CP-207 transgenic samples.

Immunogold labeling detected GFP:TGBp1 and GFP:CP in the transgenic samples. Nontransgenic samples and *Nb*COI-203 samples were included as negative controls and showed minimal labeling (Table I). Transgenic samples were also treated with buffer and secondary antisera and there was minimal labeling indicating that the TGBp1 and CP antisera specifically labeled the transgenic samples (Table I).

Immunogold labeling detected GFP:TGBp1 associated with chloroplasts (Fig. 3A) vacuoles (Fig. 3C and E), plastids (Fig. 3G), mitochondria (Fig. 3C) in companion cells. In sieve elements, immunogold label associated with the sieve plate, callose, plastids, and P-proteins (Fig 3I and K), plastids (Fig. 3G). The fixation and embedded process caused dispersal of the crystalline P-protein into fibers which were dispersed throughout the sieve elements and were gold labeled (Cronshaw and Esau, 1967). The presence of immunogold labeling on major organelles were calculated in 4-μm^2 fields and compared statistically (Table I). In companion cells, the average numbers of gold particles (of each type) associated with cytoplasm, vacuole, plasmid, mitochondria, chloroplast, plasmodesmata, and cell wall were scored.

The size ranges for the chloroplasts, vacuole, and plastids were overlapping, ranging from 1 to 16 μm, while mitochondria were between 0.4 and 0.6 μm and were significantly smaller than the other structures (Table I). For samples treated with TGBp1 antisera, gold label was highest in the chloroplast, vacuole and plastids in companion cells. In sieve elements treated with TGBp1 antisera, the average number of gold particles associating with the central cavity, plastids, P-proteins, sieve plate and callose

were scored. The greatest amount of label was associated with plastids, and minimal labeling associated with P-proteins, cytoplasm, and callose. Since the elongated bodies seen in *Nb*GFP:TGBp1-201 veins using confocal microscopy range between 30-120 μm, it is reasonable to consider that fluorescence is the agglomeration of chloroplasts, vacuoles, and plastids through the length of the companion cells, which we see labeled with TGBp1 antisera using electron microscopy.

Immunogold labeling detected GFP:CP associated with plastids, throughout the cytoplasm, embedded in the cell wall, and in starch bodies of companion cells (Fig. 4A, C, E, and G). In sieve elements, gold particle associated with plastids and were scattered throughout the central cavity (data not shown). The average number of gold particles was greatest in the cytoplasm, plastids, and cell wall in companion cells and sieve elements (Table I). There was some labeling in the sieve plate and chloroplasts.

Table I. *Distribution of immunogold labeling with TGBp1 and CP antisera in ultrastructures of companion cell and sieve elements from longitudinal and cross sections of petioles.*

Sections were treated with TGBp1, CP antisera, or buffer. Seventy 4-μm^2 fields were scored for the presence of gold particles associating with each ultrastructural element. The average number of gold particles per 4 μm^2 field area ± the standard error (SE) are reported. Treatment with TGBp1 antisera and buffer, or CP antisera and buffer were compared statistically and are indicated by capital letters. Means followed by the same capital letter are not significantly different from each other using Fisher's protected LSD procedure ($p< 0.01$). For each treatment, in each column the average numbers of gold particles associated with each ultrastructure element in companion cells or sieve elements were compared statistically. Means within a column and cell type followed by the same small letter are not significantly different from each other ($p<0.01$).

				Mean Number of Gold Particles in:					
			Size[a]	*Nb*GFP:TGBp1-201		*N. benthamiana*	*Nb*GFP:CP-207		*Nb*COI -203
Cell Type[b]	Ultrastructure	Field	(μm)	TGBp1-sera	Buffer	TGBp1-sera	CP-sera	Buffer	CP-sera
CC	Cytosol	70	ND[c]	1.70 A c	0.51 B	0.60 B	1.93 A a	0.07 B	0.11 B
	Vacuole	70	2.4-16	3.36 A b	0.24 B	0.66 B	0.01 A b	0.06 B	0.03 A
	Plastid	70	1.0-5.5	3.21 A b	0.13 B	0.61 B	2.09 A a	0.04 B	0.07 B
	Mitochondria	70	0.4-0.6	1.14 A c	0.01 B	0.04 B	0.29 A b	0.03 B	0.01 B
	Chloroplast	70	3.0-6.7	4.36 A a	0.99 B	1.29 B	0.64 A a	0.04 B	0.01 B
	Plasmodesmata	70	ND	0.36 A e	0.04 B	0.13 B	ND	ND	ND
	Cell Wall	70	2.3-3.3	0.69 A de	0.23 B	0.41 B	1.61 A a	0.11 B	0.04 B
SE	Cytoplasm	70	ND	0.80 A b	0.47 B	0.48 B	2.33 A a	0.13 B	0.13 B
	Plastid	70	1.0-5.5	4.21 A a	0.34 B	0.37 B	1.06 A b	0.09 B	0.13 B
	P-Proteins	70	0.4-1.2	0.67 A bc	0.14 B	0.14 B	ND	ND	ND
	Sieve Plate	70	ND	0.37 A c	0.07 B	0.10 B	0.50 A bc	0.0 B	0.04 B
	Callose	70	ND	0.59 A bc	0.14 B	0.03 B	0.19 A c	0.03 B	0.03 B

[a]The length of ten organelles was measured.
[b] CC, Companion cells; SE, Sieve element.
[c]ND, Not determined.

Movement Proteins Alter Xylem Development

In cross sections of leaf petioles, we discovered that the number of tracheary elements varied among the transgenic plants suggesting that the fusion proteins might impact vascular development. To test this hypothesis, we examined the effect of transgens in *Nb*GFP:TGBp1-201, *Nb*GFP:TGBp2-204, *Nb*GFP:CP-207, *Nb*GFP:GFP-201, *Nb*GFP-205 and *Nb*COI-203 petioles on xylem development (Table II). The fourth leaves from the soil surface were excised from 12-day old plants and a razor blade was used to cut petiole cross sections. Using a microscope, the numbers of tracheary elements throughout the vascular arc were counted. The numbers of tracheary elements in *Nb*GFP:GFP-201, *Nb*GFP-205 and *Nb*COI-203 plants were similar, between 35 and 38 (Table II, Fig. 6). The number of tracheary elements in *Nb*GFP:TGBp1-201 petioles were approximately 28, which is significantly less than the control *Nb*COI-203 samples. The number of tracheary elements in *Nb*GFP:CP-207 plants was significantly greater than *Nb*COI-203 at 46.7 (Table II, Fig. 6C). These data indicate the PVX TGBp1 and CP impact xylem development in these transgenic plants.

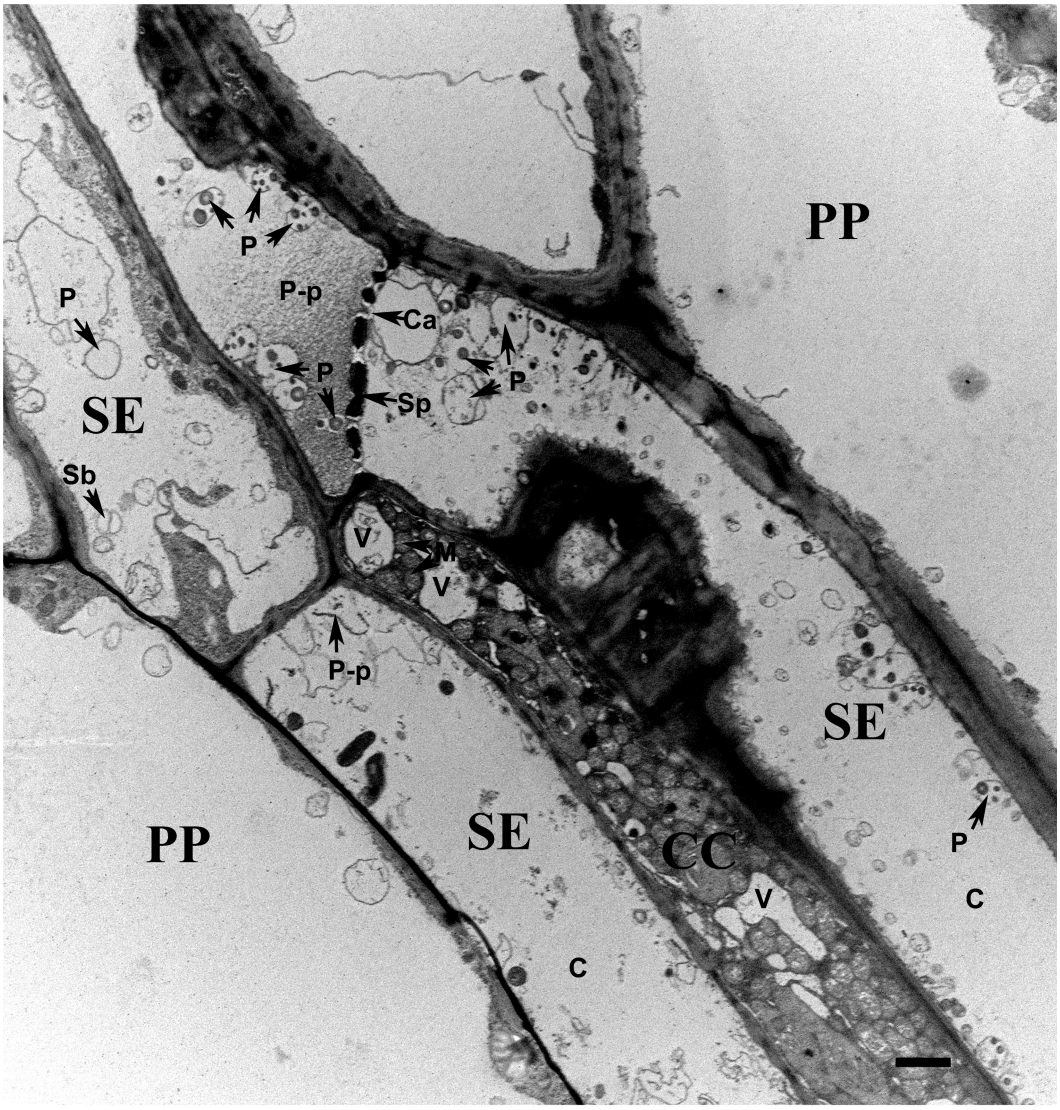


Figure 2. Electron micrograph of LR white embedded longitudinal ultrathin section of petiole showing a portion of phloem. The three major components of phloem: companion cell (CC), sieve elements (SE) and phloem parenchyma (PP) are indicated. Low magnification phloem shows numerous plastids (P), phloem proteins (P-p), a sieve plate (Sp), callose (Ca), and cytoplasm (C) within each sieve element. Within the companion cell are compacted masses of mitochondria (M). Vacuoles (V) are indicated. Bar, represent 0.5μm

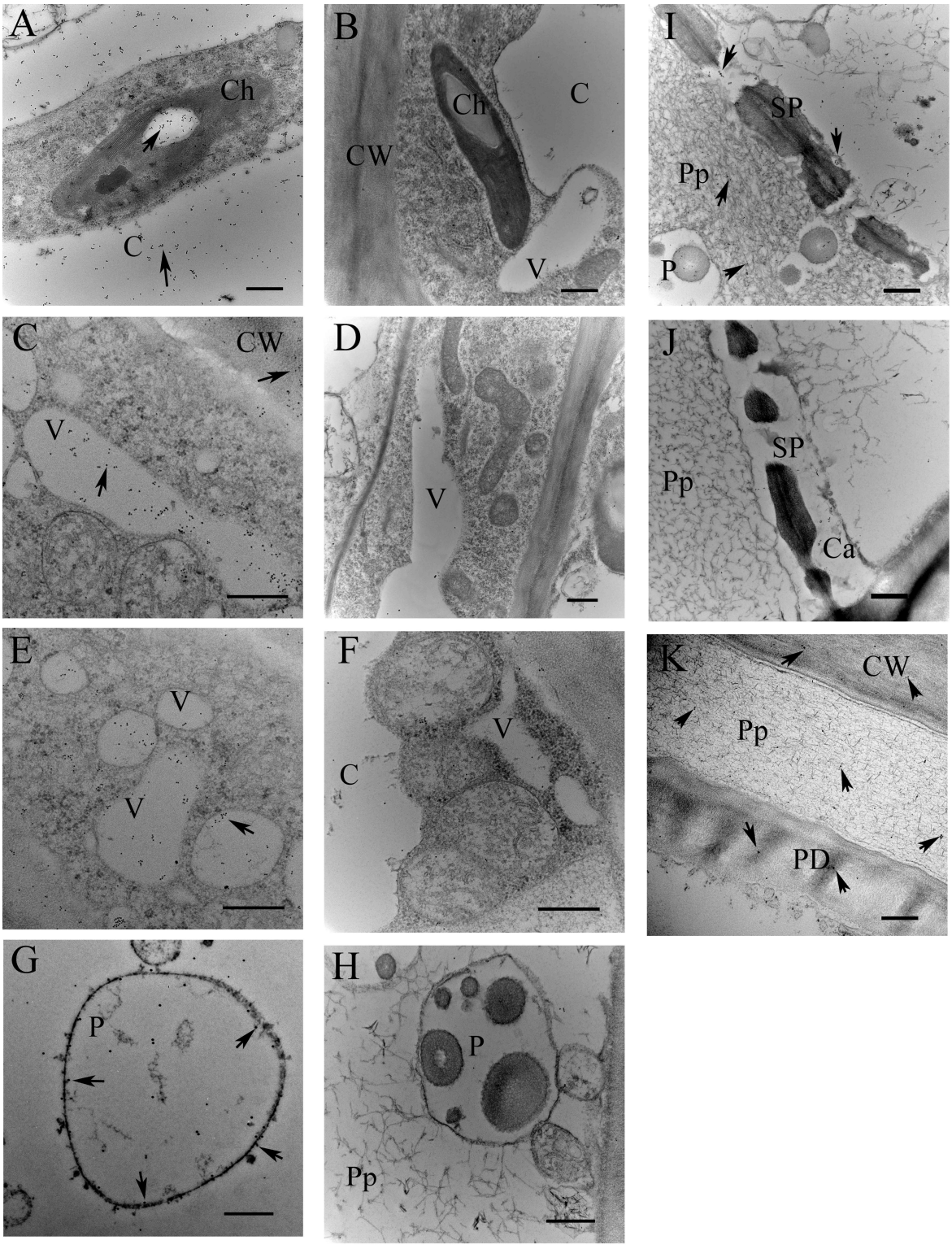


Figure 3. Electron micrographs show longitudinal sections through the vasculature of *Nb*GFP:TGBp1-207 and *Nb*GFP:TGBp1-201 petioles. A, C, E, and G, Companion cells

treated with TGBp1 antisera show gold label in chloroplasts (Ch), cytoplasm (C), large vacuole (V) plastids (P) and cell wall (CW). B, D, and F, Companion cells treated with buffer show minimal gold labeling. I and K, Sieve elements treated with TGBp1 antisera show gold label in plastids (P), dispersed phloem proteins (Pp), sieve plate (Sp) and callose (Ca). H and J, Sieve elements treated with buffer show minimal gold labeling. Bars represent 0.5μm.

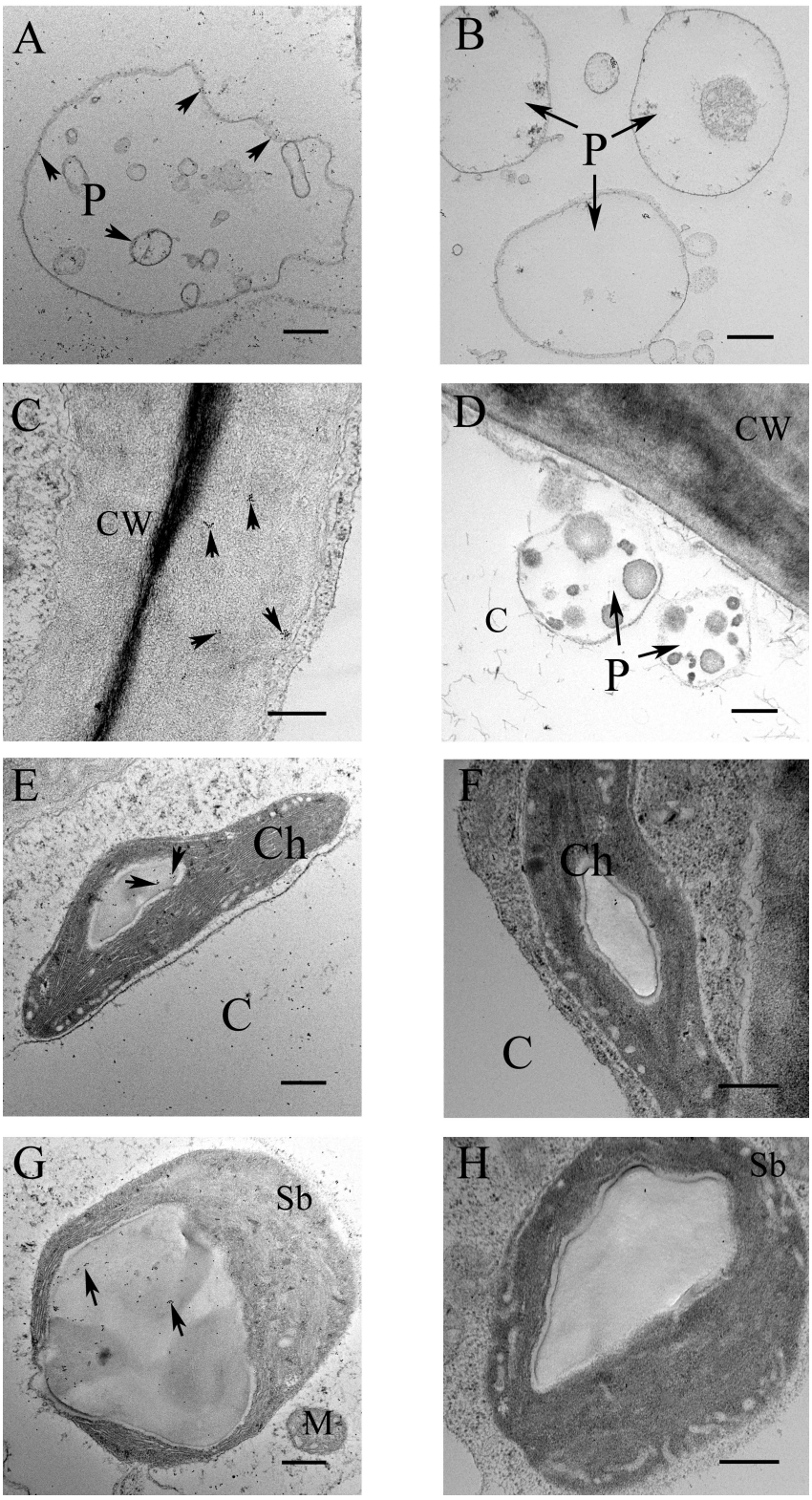


Figure 4. Electron micrographs show longitudinal sections through the vasculature of *Nb*GFP:CP-207 petioles. A, C, E, and G, Companion cells treated with CP antisera show

gold label in plastids (P), cytoplasm (C), cell walls (CW), chloroplasts (Ch), starch bodies (Sb), and mitochondria (M). B, D, and F, Companion cells show minimal gold labeling when treated with buffer and gold-conjugated antisera. Arrows indicate gold particles. Bars represent 0.5μm.

Table II. *Effects of transgenes on the number of tracheary elements in cross sections of transgenic plant petioles*

Leaves were excised from transgenic plants and the petioles were sectioned using a razor blade. Sections were placed on a slide and then stained using Toluidine blue and then studied using bright field microscopy. Toluidine blue stains the thick walls of the tracheary elements dark blue.

Transgenic plant	N[a]	Mean of tracheary elements[b]
*Nb*GFP:TGBp1-201	15	28.7 c
*Nb*GFP:TGBp2-204	15	35.7 b
*Nb*GFP:CP-207	15	46.7 a
*Nb*GFP:GFP-201	15	37.1 b
*Nb*GFP-205	15	37.9 b
*Nb*COI-203	15	35.5 b

[a] Three petiole cross sections from five plants (a total of fifteen cross sections) were scored. The numbers of tracheary elements were highly reproducible among the three cross sections per petiole.

[b]Means followed by the same letter are not significantly different from each other ($p < 0.01$).

TGBp1 Induces Starch Bodies

In *Nb*GFP:TGBp1-201, unlike *Nb*GFP:CP-207 transgenic plants or PVX-GUS infected *N. benthamiana*, there were a large number of fluorescent bodies in xylem parenchyma, pericycle and endodermal cells which resembled starch bodies (data not shown). LR-White embedded petiole cross sections of *Nb*GFP:TGBp1-201, *Nb*GFP:TGBp2-204, *Nb*GFP:CP-207, *Nb*GFP:GFP-201, *Nb*GFP-205, *Nb*COI-203 and *N. benthamiana* were treated with a solution of iodine in potassium iodide (IKI) for comparison. Only *Nb*GFP:TGBp1-201 contained large numbers of starch bodies which stained bluish-black with a solution of IKI (Cutter, 1969) (Fig 5).

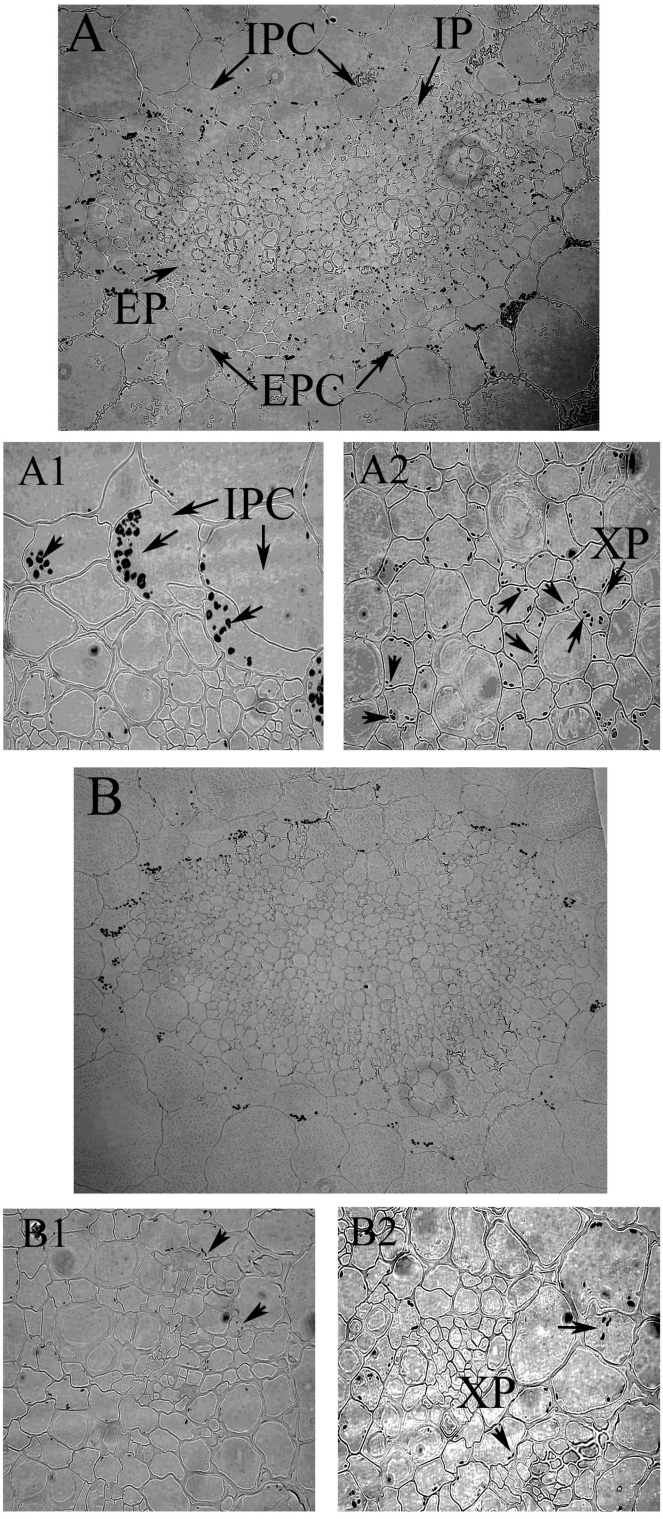


Figure 5. Images of iodine potassium iodide (IKI) treated petiole cross sections taken using bright field microscopy. Images in A and B were taken using a 10x objective and

the remaining images were taken using a 60x objective. A, Cross section from *Nb*GFP:TGBp1-201 petiole shows the vascular arc including the external pericycle (EPC) and internal pericycle (IPC). A1, At higher magnification numerous starch bodies can be seen in internal pericycle (IPC). A2, At higher magnification numerous Sbs can be seen in the xylem parenchyma (XP) and surrounding tissues. B, Cross sections of nontransgenic *N. benthamiana* show the vascular arc, external pericycle (EPC) and internal pericycle (IPC). B1, At higher magnification few starch bodies can be seen in the external phloem (EP). Arrows indicate the structures or starch bodies. B2, At higher magnification few starch bodies can be seen in the xylem parenchyma (XP) and surrounding tissues.

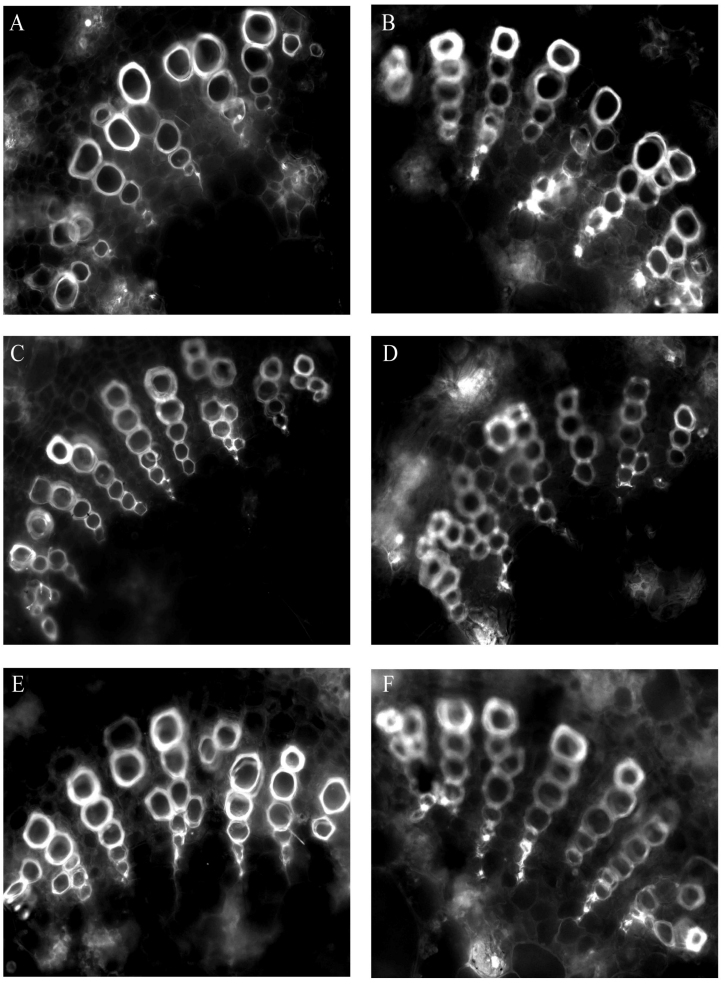


Figure 6. Microscopic images of petiole cross sections showing tracheary elements in side vascular arc. Images were taken using bright field microscopy using a 10X objective. A, *Nb*GFP:TGBp1-201; B, *Nb*GFP:TGBp2-204; C, *Nb*GFP:CP-207; D, *Nb*GFP:GFP-201; E, *Nb*GFP-205; F, *Nb*COI-203.

DISCUSSION

In this study we explored the subcellular localization of PVX TGBp1 and CP in companion cells and sieve elements using bright field, confocal, and electron microscopy. Using electron microscopy, immunogold labeling detected both GFP:TGBp1 and GFP:CP associated with plastids and chloroplasts in companion cells. In addition, GFP:TGBp1 was seen in the vacuoles of companion cells, along P-proteins and sieve plate callose deposits in the sieve elements, and associated with starch bodies in surrounding vascular tissues. GFP:CP was in the cytoplasm in the companion cells and sieve elements with minor amounts of gold label were seen along the sieve plate. Since confocal microscopy showed GFP:CP was primarily cytosolic we did not conduct further analysis of cells beyond the companion cells and sieve elements. Differences in the subcellular localization patterns of GFP:TGBp1 and GFP:CP suggests that these proteins may have some independent functions for promoting virus vascular movement and may have different impacts on disease development in the host.

GFP:TGBp1 may accumulate in the vacuole for several reasons that include: 1) storage for use later; 2) removal from the cytoplasm because it is expressed in excess; and 3) sent to the vacuole for degradation (Esau, 1965; Taiz and Zeiger, 2002; Tamura et al., 2004). While we do not know if TGBp1 accumulates in the companion cell vacuole during PVX infection, GFP:TGBp1 is expressed from the CoYMV promoter and likely accumulates in the vasculature at levels higher than in virus infected tissues. When transgenic plants were inoculated with PVX-GUS, we did not see changes in the subcellular distribution of GFP:TGBp1 indicating the fusion proteins are likely not stored in the vacuole for later use. Thus the first reason seems unlikely. Considering the second

reason, plants may respond to the fusion proteins by removing them from the cytoplasm to eliminate any potential toxic threat or effects on cellular homeostasis. This possibility seems less likely since GFP:CP was not detected in the vacuole. Thus targeting of GFP:TGBp1 to the vacuole is specific. The third possibility is that GFP:TGBp1 may be sent to the vacuole for degradation. Based on the destination selective model which describes vascular transport and exit of cellular proteins from the phloem into nonvascular tissues, proteins are degraded in non-target tissues (Aoki et al., 2005). Therefore it is reasonable to assume that GFP:TGBp1 may accumulate in the vacuole on its way to being degraded. We recently treated *Nb*GFP:TGBp1-201 and *Nb*GFP:CP-207 leaves with MG132 and MG115, which are inhibitors of the 26S proteasome and we found protein accumulation outside the veins was higher indicating that both fusion proteins are likely degraded by cellular proteases (data not shown). Interestingly, GFP:CP accumulation was greatly affected by the proteasome inhibitors while GFP:TGBp1 was mildly affected. Since the 26S proteasome degrades proteins in the cytoplasm, and GFP:CP accumulates mainly in the cytoplasm, it is likely that different protease pathways may be operating on GFP:CP and GFP:TGBp1 (data not shown). Further analysis to determine the role of protein degradation on GFP:TGBp1 and GFP:CP subcellular accumulation is currently being conducted in our laboratory.

GFP:TGBp1 and GFP:CP are both associated with plastids and chloroplasts in companion cells which sometimes serve as organelles for storing starch. Figures 4 and 5 show the chloroplasts containing starch granules and in Table II these were scored as chloroplasts. Furthermore, GFP:TGBp1 was seen in starch bodies stained with IKI solution and GFP:TGBp1 seemed to influence the number of starch bodies seen in

vascular tissues. In higher plants there are a number of enzymes which associate with starch granules regulating the composition and levels of starch within the cell(Sehnke et al., 2001). GFP:TGBp1 and GFP:CP may influence starch composition in the cell. A similar report in which the TMV movement protein was transgenically expressed in *N. tabacum* using the phloem specific rolC promoter (Almon et al., 1997), showed that starch and sucrose accumulation in source leaves was significantly higher as compared to control lines. Constitutive expression of *Tomato spotted wilt virus* (TSWV) NS_M viral movement protein using 35S promoter in *N. tabacum* was also found to increase accumulation of excessive amounts of starch in the mesophyll (Rinne et al., 2005). From expression of TMV and TSWV movement proteins in transgenic *N. tabacum* we learn that viral movement proteins can alter starch accumulation in transgenic plants. However, those studies did not indicate whether the increase in starch accumulation is due to proliferation of starch bodies or increased accumulation of starch in pre-existing starch bodies. Our data therefore further advance the previous observation of our research colleagues that the stress induced by movement proteins in transgenic plant alters starch allocation in favour of particular organ by proliferation of more starch bodies.

In sieve elements GFP:TGBp1 and GFP:CP associated with plastids and the cytoplasm, as in companion cells. Interestingly, GFP:TGBp1 was also seen to associate with dissociated P-protein fibers (Table I and Fig 3K). P-proteins oscillate between crystalloid and dispersed conformations. In this study we detected crystalloid P-proteins (data not shown) but did not observe the dispersed conformation which resembles a round body forming a plug at the sieve plate (Knoblauch et al., 2001; Cillo et al., 2002).

Since we failed to detect crystalloid or dispersed P-proteins in the control samples, it is possible that the P-protein seen in *Nb*GFP:TGBp1-201 sieve elements may have been dissolved into individual fibers as the result of poor fixation and LR-White embedding (Cronshaw and Esau, 1967, , 1978). Alternatively the PVX TGBp1 caused the P-proteins to dissolve into individual fibers. Dispersal and plug formation are seen in experiments in response to plasma membrane leakage caused by wounding or by introduced changes in turgor pressure with in sieve element (Golecki et al., 1999; Knoblauch et al., 2001; Kehr, 2006). Calcium fluxes are induced as the result of wounding and an influx of Ca^{2+} into the sieve elements was shown to trigger crystalloid P-protein dispersal and plug formation as well as enhanced callose deposition at the sieve plate. The combined plug and callose reduce hydraulic resistance and prevent leakage of phloem contents and reduce phloem translocation (Cronshaw and Esau, 1978; Knoblauch et al., 2001). While there is no evidence whether P-proteins play a role in host defenses to viral pathogens, it is worth speculating that TGBp1 may function to block plug formation and thereby enable long distance viral translocation to occur unheeded. GFP:TGBp1 association with callose at the sieve plate may prevent further build up of callose or may prevent the plug from lodging in the sieve plate. If sieve plate pores are plugged by P-proteins PVX vascular spread would be obstructed and vascular spread of the virus would be limited. Therefore PVX may employ a unique strategy to suppress defenses at the interface of sieve plates. In this study since we observed TGBp1 to associate with dissociated P-proteins fibers but we do not know yet such association is a strategy used by PVX to suppress the host defense and facilitate its translocation in the phloem. Further study is

needed to compare the nature of P-proteins in virus infected and non-infected transgenic and nontransgenic plants.

Cucumber mosaic virus 1a protein is the only viral protein reported to be associate with dispersed P-proteins, (Cillo et al., 2002). Using electron microscopy, researchers showed that the 1a protein associated with the dispersed P-protein plug neighboring sieve plates (Cillo et al., 2002). Our experiments using transgenic *Nb*GFP:TGBp1-201 plants differ from the study of CMV infected plants in that we did not detect the dispersed conformation. However, our experiments were conducted using transgenic leaf petioles and while the CMV experiments looked at 1a protein accumulation in the minor veins of infected leaves. Thus it is possible that different P-protein conformations may be dominant and easier to observe in the different organs. Alternatively, transgenic expression of the PVX TGBp1 protein may have had an effect on the P-protein conformation or import into the sieve elements from companion cells during plant development that is not seen in virus infected tissues. For example, if PVX TGBp1 binds P-proteins in companion cells, it is possible that it may affect movement across the plasmodesmata or assembly into the crystalloid P protein bodies.

Crystalloid P-proteins are composed of PP1 and PP2 proteins. PP1 and PP2 also exist and monomers and dimmers which move through the sieve tube translocation stream (Cronshaw and Esau, 1967; Steve and Northcote, 1983; Golecki et al., 1999) and are thought to modulate the flow of assimilates through the phloem independent of the P-protein bodies. A number of PP1- and PP2-like proteins have been isolated from phloem exudates of several angiosperm species (Dinant et al., 2003; Kehr, 2006). In general, P-protein monomer and dimmers have been described as chaperones for the translocation of

proteins and nucleic acids through the phloem (Golecki et al., 1999; Gomez et al., 2005). PP2-like proteins such as CmmPP2, CsPP2, CmmLec17 have been characterized as chaperones which assist phloem translocation of cellular, viroid, and viral RNAs (Gomez and Pallas, 2004; Gomez et al., 2005). In this study we observed TGBp1 associated with P-proteins suggesting TGBp1 may bind P-proteins to chaperone movement through the phloem. Other plant viral proteins known to associate with P-proteins include the CMV 1a protein (Cillo et al., 2002). Further research is needed to determine if TGBp1 interacts with P-protein monomers or dimmers to know if this interaction is necessary for virus translocation through the phloem.

Several studies showed that transgenically expressing viral movement proteins have effects on development and physiology of plants (Almon et al., 1997; Kobayashi et al., 2004; Rinne et al., 2005). In our study the number of xylem strands seen in petiole cross sections was reduced in *Nb*GFP:TGBp1-201 and was increased in *Nb*GFP:CP-207 plants relative to the control lines. In a previous study, Kobayashi et al., (2004) reported that PVX TGBp1-transgenic *N. tabacum* plants were stunted in comparison to nontransgenic plants. These combined studies suggest that TGBp1 may be responsible for the disease phenotype seen in PVX infected plants.

LITERATURES CITED

Ajjikuttira P, Loh CS, Wong SM (2005) Reciprocal function of movement proteins and complementation of long-distance movement of *Cymbidium mosaic virus* RNA by *Odontoglossum ringspot virus* coat protein. J Gen Virol **86:** 1543-1553

Almon E, Horowitz M, Wang HL, Lucas WJ, Zamski E, Wolf S (1997) Phloem-specific expression of the *Tobacco Mosaic Virus* movement protein alters carbon metabolism and partitioning in transgenic potato plants. Plant Physiol **115:** 1599-1607

Aoki K, Suzui N, Fujimaki S, Dohmae N, Yonekura-Sakakibara K, Fujiwara T, Hayashi H, Yamaya T, Sakakibara H (2005) Destination-selective long-distance movement of phloem proteins. Plant Cell **17:** 1801-1814

Berlyn GP, Miksche JP (1976) Botanical microtechnique and cytochemistry. The Iowa State University Press Ames, Iowa

Carrington JC, Jensen PE, Schaad MC (1998) Genetic evidence for an essential role for potyvirus CI protein in cell-to-cell movement. Plant J **14:** 393-400

Carrington JC, Kasschau KD, Mahajan SK, Schaad MC (1996) Cell-to-cell and long-distance transport of viruses in plants. Plant Cell **8:** 1669-1681

Cillo F, Roberts IM, Palukaitis P (2002) In situ localization and tissue distribution of the replication-associated proteins of *Cucumber mosaic virus* in tobacco and cucumber. J. Virol. **76:** 10654-10664

Citovsky V, McLean BG, Zupan JR, Zambryski P (1993) Phosphorylation of *Tobacco mosaic virus* cell-to-cell movement protein by a developmentally regulated plant cell wall-associated protein kinase. Genes Dev **7:** 904-910

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

Cronshaw J, Esau K (1967) Tubular and fibrillar components of mature and differentiating sieve elements. J. Cell Biol. **34:** 801-815

Cronshaw J, Esau K (1978) P protein in the phloem of cucurbita. Ann Bot **42:** 353-361

Cutter EG (1969) Plant anatomy: experiment and interpretation. Edward Arnold (Publishers) Ltd. London, UK, London

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

Dinant S, Clark AM, Zhu Y, Vilaine F, Palauqui J-C, Kusiak C, Thompson GA (2003) Diversity of the superfamily of phloem lectins (Phloem Protein 2) in Angiosperms. Plant Physiol. **131:** 114-128

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

Driskel BA, Doss P, Littlefield LJ, Walker NR, Verchot LJ (2004) *Soilborne Wheat mosaic virus* movement protein and RNA and *Wheat spindle streak mosaic virus* coat protein accumulate inside resting spores of their vector, *Polymyxa graminis*. Mol Plant Microbe Interact **17:** 739-748

Esau K (1965) Plant anatomy, Ed 2nd. John Wiley & Sons, Inc., New York

Esau K (1973) Comparative structure of companion cells and phloem parenchyma cells in *Mimosa pudica* L. Ann Bot **37:** 625-632

Evert RF (1982) Sieve-Tube structure in relation to function. BioScience **32:** 789-795

Fisher DG (1991) Plasmodesmatal frequency and other structural aspects of assimilate collection and phloem loading in leaves of *Sonchus oleraceus* (Asteraceae), a species with minor vein transfer cells. Am J Bot **78:** 1549-1559

Gilbertson RL, Lucas WJ (1996) How do viruses traffic on the 'vascular highway'? Trends Plant Sci **1:** 260-268

Golecki B, Schulz A, Thompson G (1999) Translocation of structural P proteins in the phloem. Plant Cell **11:** 127-140

Gomez G, Torres H, Pallas V (2005) Identification of translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system. Plant J **41:** 107-116

Howard AR, Heppler ML, Ju HJ, Krishnamurthy K, Payton ME, Verchot-Lubicz J (2004) *Potato virus X* TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves. Virology **328:** 185-197

Huisman MJ, Linthorst HJ, Bol JF, Cornelissen JC (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

Itaya A, Hickman H, Bao Y, Nelson R, 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, Ma F, Qi Y, Matsuda Y, Zhu Y, Liang G, Ding B (2002) Plasmodesma-mediated selective protein traffic between "symplasmically isolated" cells probed by a viral movement protein. Plant Cell **14:** 2071-2083

Ju HJ, Brown JE, Ye CM, Verchot-Lubicz J (2007) Mutations in the central domain of *Potato virus X* TGBp2 eliminate granular vesicles and virus cell-to-cell trafficking. J Virol **81:** 1899-1911

Ju HJ, Samuels TD, Wang YS, Blancaflor E, Payton M, Mitra R, Krishnamurthy K, Nelson RS, Verchot-Lubicz J (2005) The *Potato virus X* TGBp2 movement protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol **138:** 1877-1895

Karpova OV, Ivanov KI, Rodionova NP, Dorokhov Yu L, Atabekov JG (1997) Nontranslatability and dissimilar behavior in plants and protoplasts of viral RNA and movement protein complexes formed in vitro. Virology **230:** 11-21

Kehr J (2006) Phloem sap proteins: their identities and potential roles in the interaction between plants and phloem-feeding insects. J. Exp. Bot. **57:** 767-774

Knoblauch M, Peters WS, Ehlers K, van Bel AJE (2001) Reversible calcium-regulated stopcocks in legume sieve tubes. Plant Cell **13:** 1221-1230

Kobayashi K, Sarrobert C, Ares X, Rivero MM, Maldonado S, Robaglia C, Mentaberry A (2004) Over-expression of potato virus X TGBp1 movement

protein in transgenic tobacco plants causes developmental and metabolic alterations. Plant Physiol Bioch **42:** 731-738

Krishnamurthy K, Heppler M, Mitra R, Blancaflor E, Payton M, Nelson RS, 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

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

Lawrence DM, Jackson AO (2001) Interactions of the TGB1 protein during cell-to-cell movement of *Barley stripe mosaic virus*. J Virol **75:** 8712-8723

Lazarowitz SG, Beachy RN (1999) Viral movement proteins as probes for intracellular and intercellular trafficking in plants. Plant Cell **11:** 535-548

Lough TJ, Netzler NA, Emerson SJ, Sutherland P, Carr F, Beck DL, Lucas WJ, Forster RL (2000) Cell-to-cell movement of potexviruses: evidence for a ribonucleic protein complex involving the coat protein and first triple gene block protein. Mol Plant Microbe Interact **13:** 13:962–974

Lucas WJ, Wolf S (1999) Connections between virus movement, macromolecular signaling and assimilate allocation. Curr Opin Plant Biol **2:** 192-197

Mezitt LA, Lucas WJ (1996) Plasmodesmal cell-to-cell transport of proteins and nucleic acids. Plant Mol Biol **32:** 251-273

Mitra R, Krishnamurthy K, Blancaflor E, Payton M, Nelson RS, 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:** 35-48

Morozov SY, Solovyev AG (2003) Triple gene block: modular design of a multifunctional machine for plant virus movement. J Gen Virol **84:** 1351-1366

Oparka KJ, Turgeon R (1999) Sieve elements and companion cells-traffic control centers of the phloem. Plant Cell **11:** 739-750

Parthasarathy MV, Larry HK (1976) Palm "wood". Wood Sci Technol **10:** 247-271

Reichel C, Beachy RN (2000) Degradation of *Tobacco mosaic virus* movement protein by the 26S proteasome. J. Virol. **74:** 3330-3337

Rinne PLH, Boogaard Rvd, Mensink MGJ, Kopperud C, Kormelink R, Goldbach R, Schoot Cvd (2005) Tobacco plants respond to the constitutive expression of the tospovirus movement protein NSM with a heat-reversible sealing of plasmodesmata that impairs development. Plant J **43:** 688-707

Rojas MR, Jiang H, Salati R, Xoconostle-Cazares B, Sudarshana MR, Lucas WJ, Gilbertson RL (2001) Functional analysis of proteins involved in movement of the monopartite begomovirus, *Tomato yellow leaf curl virus*. Virology **291:** 110-125

Santa Cruz S, Chapman S, Roberts AG, Roberts IM, Prior DA, Oparka KJ (1996) Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. Proc Natl-Acad Sci **93:** 6286-6290

Sehnke PC, Chung H-J, Wu K, Ferl RJ (2001) Regulation of starch accumulation by granule-associated plant 14-3-3 proteins. Proc Nati-Acad Sci **98:** 765-770

Sjolund RD (1997) The phloem sieve element: A river runs through it. Plant Cell **9:** 1137-1146

Solovyev AG, Stroganova TA, Zamyatnin AA, Jr., Fedorkin ON, Schiemann J, Morozov SY (2000) Subcellular sorting of small membrane-associated triple gene block proteins: TGBp3-assisted targeting of TGBp2. Virology **269:** 113-127

Spillane C, Verchot J, Kavanagh TA, Baulcombe DC (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:** 76-84

Steve M, Northcote D (1983) Subunit structure and interactions of the phloem proteins of *Cucurbita maxima* (Pumpkin). Eur. J. Biochem **134:** 561-569

Taiz L, Zeiger E (2002) Plant physiology, Ed 3 rd. Sinauer Associate, inc., Sunderland, MA

Tamai A, 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. Mol Plant Microbe Interact **14:** 1158-1167

Tamura K, Yamada K, Shimada T, Hara-Nishimura I (2004) Endoplasmic reticulum-resident proteins are constitutively transported to vacuoles for degradation. Plant J **39:** 393-402

Toth RL, Chapman S, Carr F, Santa Cruz S (2001) A novel strategy for the expression of foreign genes from plant virus vectors. FEBS Lett **489:** 215-219

Verchot J, Driskel BA, Zhu Y, Hunger RM, Littlefield LJ (2001) Evidence that *Soilborne wheat mosaic virus* moves long distance through the xylem in wheat. Protoplasma **218:** 57-66

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

VITA

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Dissertation: A STUDY OF VASCULAR MOVEMENT OF POTATO VIRUS X (PVX) TGBp1, TGBp2 AND CP USING THE COMMELINA YELLOW MOTLE VIRUS PROMOTER AND GFP

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Title of Study: A STUDY OF VASCULAR MOVEMENT OF POTATO VIRUS X (PVX) TGBp1, TGBp2 AND CP USING THE COMMELINA YELLOW MOTTLE VIRUS PROMOTER AND GFP

Pages in Study: 153 Candidate for the Degree of Doctor of Philosophy

Major Field: Plant Pathology

Scope and Method of Study:

The green fluorescent protein (GFP) was fused to *Potato virus X* (PVX) TGBp1, TGBp2, or coat protein (CP) genes and inserted into the plasmids next to a companion cell specific CoYMV promoter. These plasmids were used to prepare transgenic tobacco plants expressing PVX proteins specifically in the phloem. Transgenic tobacco plants expressing a GFP:GFP fusion were also prepared as a control. GFP fluorescence was used to visualize phloem translocation and exit of the fusion proteins. Immunofluorescence and immunogold labeling was used to characterize the sub-cellular distribution of the fusion proteins within phloem.

Findings and Conclusions:

Fluorescence due to GFP:TGBp1, GFP:TGBp2, GFP:CP, and GFP:GFP was seen in transgenic tobacco leaf veins and failed to spread to nonvascular tissue. However, following PVX inoculation of GFP:TGBp1 and GFP:CP transgenic plants fluorescence spread into nonvascular tissues. Thus the destination of GFP:TGBp1 and GFP:CP, but not GFP:TGBp2 and GFP:GFP was altered by the presence of the virus indicating that the TGBp1 and CP directly interact with the virus to unload from the veins. Further studies explored phloem translocation of proteins within the stem and petioles. In petiole cross sections all fusion proteins were seen in phloem companion cells, sieve elements, and phloem parenchyma indicating that the proteins spread from the companion cells into surrounding phloem tissues. Only GFP:TGBp1 was seen in xylem parenchyma indicating that TGBp1 has a specific ability to move extensively throughout the vasculature. Nontransgenic scions were grafted to transgenic rootstocks and after 28 days fluorescence was seen in scions indicating that all fusions were phloem mobile.

Immunogold labeling and transmission electron microscopy revealed GFP:TGBp1 and GFP:CP accumulated in the chloroplasts, plastids, vacuoles and cytoplasm in companion cells, and associated with plastids and P-proteins in sieve elements. GFP:TGBp1 expressing plants showed increased numbers of starch bodies in xylem parenchyma, pericycles and endodermal cells and fewer tracheary elements in petiole cross sections suggesting that TGBp1 affects vascular development and carbohydrate partitioning.

ADVISER'S APPROVAL: Dr. Jeanmarie Verchot-Lubicz