

Potato virus X TGBp1 induces plasmodesmata gating and moves between cells in several host species whereas CP moves only in *N. benthamiana* leaves

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Abstract

Experiments were conducted to compare the plasmodesmal transport activities of *Potato virus X* (PVX) TGBp1 and coat protein (CP) in several plant species. Microinjection experiments indicated that TGBp1 gates plasmodesmata in *Nicotiana tabacum* leaves. These results support previous microinjection studies indicating that TGBp1 gates plasmodesmata in *Nicotiana benthamiana* and *Nicotiana clevelandii* leaves. To study protein movement, plasmids expressing the green fluorescent protein (GFP) gene fused to the PVX TGBp1 or CP genes were biolistically bombarded to leaves taken from four different PVX host species. GFP/TGBp1 moved between adjacent cells in *N. tabacum*, *N. clevelandii*, *N. benthamiana*, and *Lycopersicon esculentum*, whereas GFP/CP moved only in *N. benthamiana* leaves. Mutations m12 and m13 were introduced into the TGBp1 gene and both mutations eliminated TGBp1 ATPase active site motifs, inhibited PVX movement, reduced GFP/TGBp1 cell-to-cell movement in *N. benthamiana* leaves, and eliminated GFP/TGBp1 movement in *N. tabacum*, *N. clevelandii*, and *L. esculentum* leaves. GFP/TGBp1m13 formed aggregates in tobacco cells. The ability of GFP/CP and mutant GFP/TGBp1 fusion proteins to move in *N. benthamiana* and not in the other PVX host species suggests that *N. benthamiana* plants have a unique ability to promote protein intercellular movement.

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Introduction

Potexviruses require the triple gene block (TGB) proteins and the viral coat protein (CP) to mediate virus cell-to-cell movement (Forster et al., 1992; Gilmer et al., 1992; Huisman et al., 1988). The *Potato virus X* (PVX) movement proteins are named TGBp1, TGBp2, and TGBp3, and have molecular masses of 25-, 12-, and 8-kDa, respectively. The TGB proteins are highly conserved among members of the genera *Potexvirus*, *Hordeivirus*, *Benyvirus*, and *Carlavirus* (Gilmer et al., 1992; Memelink et al., 1990; Morozov et al.,

1987; Skryabin et al., 1988). TGBp1 was described as a member of the superfamily I RNA helicases based on amino acid sequence analyses (Morozov et al., 1999). Potexvirus and hordeivirus TGBp1 proteins were shown in vitro to have RNA binding, ATPase, and helicase activities (Donald et al., 1997; Kalinina et al., 2001, 2002; Rouleau et al., 1994). The potexviruses TGBp2 and TGBp3 have transmembrane domains and associate with the endoplasmic reticulum (Krishnamurthy et al., 2003; Mitra et al., 2003). It is likely that the TGB proteins interact with each other to facilitate virus movement; however, the nature of these interactions is poorly understood.

There has been some confusion in the literature as to which potexvirus proteins induce plasmodesmata gating or move through plasmodesmata. Some reports indicate

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that TGBp1 is the primary viral factor that gates and moves through plasmodesmata while other reports suggest that TGBp2 is primarily responsible for plasmodesmata gating. In two microinjection studies, the potexvirus TGBp1 was identified to be the viral factor responsible for increasing the plasmodesmal size exclusion limit (SEL) for virus cell-to-cell movement (Angell et al., 1996; Lough et al., 1998). In the first study, F-dextrans of various sizes were injected into *Nicotiana clevelandii* trichome cells along with either wild-type PVX virions or virions of a mutant PVX which had a large segment of TGBp1 deleted (Angell et al., 1996). Wild-type PVX, but not the mutant PVX, increased the plasmodesmal SEL to allow movement of large dextrans between adjacent cells (Angell et al., 1996). In the second study, F-dextrans of various sizes were injected into mesophyll cells of transgenic *Nicotiana benthamiana* expressing the TGB proteins of *White clover mosaic virus* (WCIMV). WCIMV TGBp1, but not TGBp2 or TGBp3, increased the plasmodesmal SEL for transport of large dextrans (Lough et al., 1998). In a third microinjection study using PVX CP-expressing transgenic *Nicotiana tabacum* leaves, it was reported that the PVX CP did not increase the plasmodesmal SEL to allow movement of large F-dextrans (Oparka et al., 1996).

It was recently reported that TGBp2 was responsible for increasing the plasmodesmal SEL (Fridborg et al., 2003; Tamai and Meshi, 2001). Plasmids expressing the green fluorescent protein gene (GFP) fused to the TGBp2 gene were biolistically delivered to *N. benthamiana* leaves and protein cell-to-cell movement was observed. Because these results seem to conflict with previous findings relating to TGBp1, we have undertaken a series of experiments to determine which of the PVX proteins move from cell to cell. Plasmids expressing GFP/TGBp2 or GFP/TGBp3 were biolistically delivered to six different PVX host species to compare protein movement in a broader range of PVX host plants (Krishnamurthy et al., 2003). GFP/TGBp2 and GFP/TGBp3 each moved from cell to cell in *N. benthamiana* leaves but were restricted primarily to single cells in *N. tabacum*, *N. clevelandii*, *Lycopersicon esculentum*, *Chenopodia quinoa*, and *Gomphrena glaberrima* (Krishnamurthy et al., 2003).

The goal of this study is to determine if PVX TGBp1 and CP increase the plasmodesmal SEL and move from cell to cell in several PVX host species. TGBp1-expressing transgenic *N. tabacum* leaves (Verchot et al., 1998) were used in microinjection studies to determine if TGBp1 can induce changes in the plasmodesmal SEL in *N. tabacum*. These microinjection studies were conducted to complement previous studies in *N. benthamiana* and *N. clevelandii*, and to provide more information about PVX TGBp1 activities in various host plants. In addition, the ability of PVX TGBp1 and CP to move from cell to cell was studied in *N. tabacum*, *N. benthamiana*, *N. clevelandii*, and *L. esculentum* leaves. Mutations were intro-

duced into the TGBp1 NTPase active site and the effects of these mutations on protein movement were host dependent. However, when the same mutations were introduced into the PVX genome, virus movement was eliminated in all host species tested.

Results

TGBp1 increases the plasmodesmal SEL in N. tabacum leaves

It was determined using microinjection techniques that the typical plasmodesmal SEL is below 1 kDa in tobacco leaves (Robards and Lucas, 1990). The low molecular weight (457 Da) Lucifer Yellow CH dilithium salt (LYCH) is a fluorescent dye that can pass through plasmodesmata, whereas F-dextrans of 4.4- or 9.5-kDa are often restricted to single cells in tobacco leaves (Ding et al., 1992; Wolf et al., 1989). TGBp1 was reported to increase the plasmodesmal SEL in *N. clevelandii* and *N. benthamiana* leaves, allowing movement of the large F-dextrans between adjacent cells (Angell et al., 1996; Lough et al., 1998).

TGB100 transgenic *N. tabacum* plants express the PVX TGBp1 and were used to determine if TGBp1 increases the plasmodesmal SEL in *N. tabacum* plants as in other *Nicotiana* species. The TGB100 plants were selected for these experiments because they are able to complement cell-to-cell movement of TGBp1-deficient PVX viruses (Verchot et al., 1998). The 4.4- or 9.5-kDa F-dextrans were injected into mesophyll cells of nontransgenic and TGB100 transgenic *N. tabacum* source leaves, and intercellular movement of the fluorescent-labeled molecules was recorded. LYCH was injected into nontransgenic and TGB100 transgenic mesophyll cells as a control to ensure plasmodesmata were not closed as a result of tissue preparation.

Fig. 1 shows representative images collected within 3–5 min after injection of LYCH, 4.4-kDa F-dextrans, or 9.5-kDa F-dextrans in nontransgenic and TGB100 leaves. LYCH moved out from 84% to 85% of injected cells in nontransgenic and TGB100 leaves, respectively (Table 1). The 4.4-kDa F-dextrans moved out from 29% of the injected cells in nontransgenic leaves. Movement of the 9.5-kDa F-dextrans was not observed in nontransgenic leaves (Table 1). In TGB100 transgenic *N. tabacum*, the percentages of cells allowing movement of 4.4- and 9.5-kDa F-dextrans were 79% and 69%, respectively (Table 1). Thus, the transgenically expressed TGBp1 increased the plasmodesmal SEL to allow movement of large F-dextrans between adjacent mesophyll cells. Evidence presented in this and previous microinjection studies indicates that potexvirus TGBp1 increases the plasmodesmal SEL in *N. tabacum*, *N. benthamiana*, and *N. clevelandii* plants (Angell et al., 1996; Lough et al., 1998).

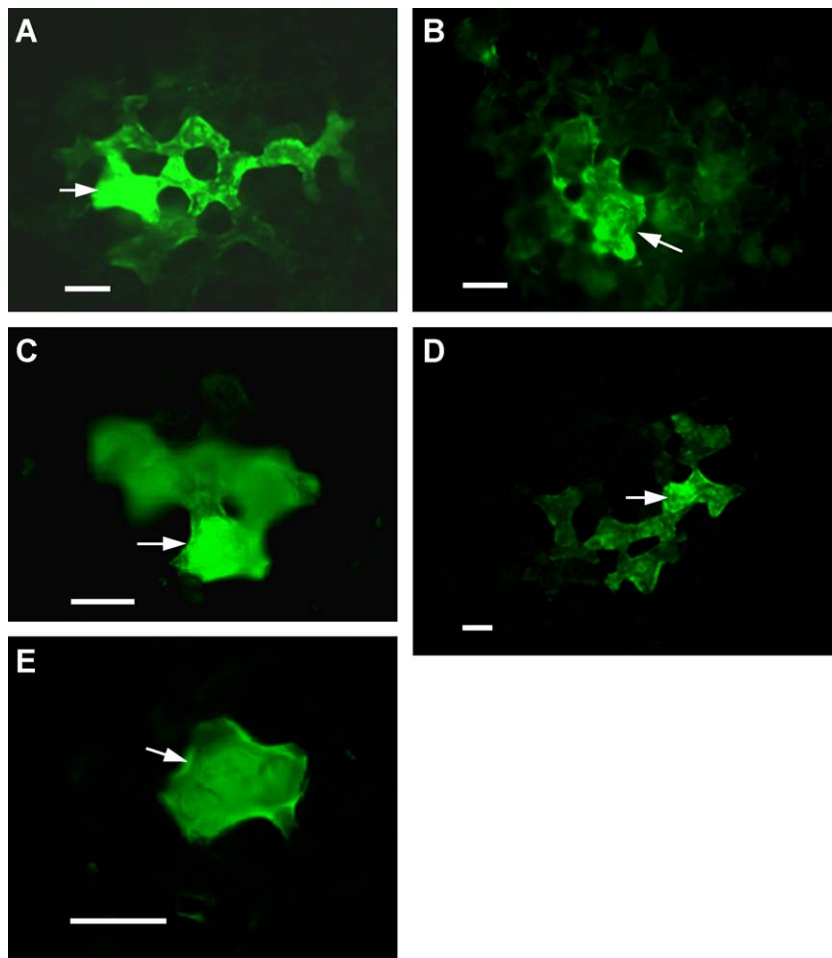


Fig. 1. Images of fluorescence in mesophyll cells following injection of LYCH or F-dextran dyes. Arrowheads in each panel indicate cells directly injected with dye or F-dextrans. Panels (A) and (B) show extensive spread of LYCH fluorescence between mesophyll cells in nontransgenic and TGB100 transgenic leaves, respectively. Panels (C) and (D), respectively, show cell-to-cell movement of 4.4-kDa F-dextrans in nontransgenic leaves and 9.5-kDa F-dextrans in TGB100 leaves. In panel (E), 9.5-kDa dextran is restricted to single cells in nontransgenic leaves. Scale bars = 40 μ m.

GFP was fused to PVX TGBp1 and CP to visualize plasmodesmata transport

The ability of PVX TGBp1 and CP to move from cell to cell was studied in several host species. The PVX CP was reported to move from cell to cell in *N. benthamiana* but was primarily restricted to single cells in *N. tabacum* leaves (Krishnamurthy et al., 2002). Thus, we do not know if PVX CP preferentially moves between cells in most host species

Table 1
Movement of fluorescent dyes between mesophyll cells in leaves of nontransgenic or TGB100 transgenic *N. tabacum*.^a

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

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

or if observations made in *N. benthamiana* plants are unique. Resolving these different observations is important for building an accurate model describing which PVX proteins mediate plasmodesmata transport of the virus.

To determine if cell-to-cell movement is an intrinsic property of PVX TGBp1 or CP, pRTL2 plasmids expressing GFP/TGBp1 or-GFP/CP fusion proteins were used (Fig. 2 and Table 2). The GFP/CP fusion is derived from the pPVX.GFP-CP infectious clone, which was obtained from Dr. Simon Santa Cruz (Horticulture International, Wellbourne, UK). The pPVX.GFP-CP infectious clone contains GFP fused to the PVX CP ORF. The 2A peptide of *Foot and mouth disease virus* (FMDV) is inserted between the GFP and CP domains and provides partial proteolytic processing (Santa Cruz et al., 1996). The resulting PVX virions are fluorescent particles coated with GFP (Santa Cruz et al., 1996). This has been a valuable tool for studying PVX CP functions during virus infection and cell-to-cell movement of PVX (Krishnamurthy et al., 2002; Santa Cruz et al., 1996). Plasmids expressing GFP alone were used as controls. In addition, two mutations m12 and m13 were

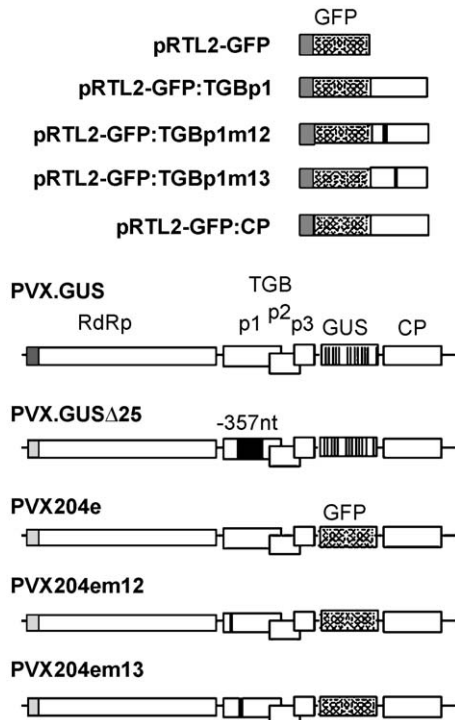


Fig. 2. Schematic representation of pRTL2 constructs and PVX infectious clones. GFP (hatched box) and the GFP fused genes are adjacent to the CaMV 35S promoter (light gray box). PVX TGBp1 and CP ORFs are indicated by open boxes. Mutations m12 and m13 are indicated by vertical lines in GFP/TGBp1 coding sequences and in the PVX genome. PVX.GUS plasmids contain T7 promoters (dark gray box) while PVX.GUS Δ 25 and each PVX204e derivative contain CaMV 35S promoters (Baulcombe et al., 1995; Chapman et al., 1992). Within the infectious clones, all PVX ORFs are represented as open boxes and lines indicate untranslated sequences. The name for each infectious clone is indicated on the left above the diagram and the names for each PVX ORF are indicated at the top. Both GUS (box with vertical lines) and GFP were inserted into plasmids under the control of a duplicated subgenomic RNA promoter (Baulcombe et al., 1995; Chapman et al., 1992). Deletion of 357 nt in PVX.GUS Δ 25 is indicated by a black box within the TGBp1 coding region.

separately introduced into pRTL2-GFP/TGBp1 plasmids (Fig. 2). The m12 and m13 mutations are substitution mutations replacing the GKS and DEY motifs with

sequences encoding AET and RRF, respectively. TGBp1 is an RNA helicase and has two amino acid sequence motifs GKS and DEY that provide ATP hydrolysis (Gorbalenya and Koonin, 1993; Morozov et al., 1999).

Plasmids were bombarded to *N. benthamiana*, *N. tabacum*, *N. clevelandii*, and *L. esculentum* source leaves, and movement of fluorescence was monitored at 1 day post bombardment (dpb). Fluorescence was observed either in clusters of adjacent cells when GFP/TGBp1 or GFP/CP moved from cell to cell, or in single cells when proteins were restricted to single cells. The proportions of cell clusters containing fluorescence due to GFP, GFP/TGBp1, GFP/TGBp1m12, GFP/TGBp1m13, or GFP/CP were compared statistically for each plant species tested. For each plasmid, statistical comparisons were also conducted among all plant species (Table 2). The accumulation of proteins in multiple cell clusters was determined to be the result of an active cell-to-cell movement mechanism when the proportions of multiple cell clusters due to each fusion protein(s) were significantly different from the proportions due to the control GFP (which does not move from cell to cell).

GFP fluorescence was detected primarily in single cells in *N. tabacum*, *N. clevelandii*, *N. benthamiana*, and *L. esculentum* source leaves bombarded with pRTL2-GFP plasmids (Fig. 3 and Table 2). GFP lacks the signals for active transport through plasmodesmata and is unable to diffuse into adjacent cells in source leaves (Itaya et al., 1997; Krishnamurthy et al., 2002; Mitra et al., 2003; Morozov et al., 1997; Yang et al., 2000). GFP was detected in two or more adjacent cells on rare occasions (between 2.3% and 6.8% of sites viewed in bombarded leaves) (Table 2) and likely resulted from simultaneous introduction of pRTL2-GFP plasmids into neighboring cells. When statistical comparisons were made among plant species, GFP performed the same in all plant species (Table 2). There were no significant differences between plant species in the proportions of sites containing GFP in cell clusters (Table 2; $P < 0.05$).

GFP/TGBp1 was observed in multiple cell clusters in *N. tabacum*, *N. clevelandii*, *N. benthamiana*, and *L. esculentum* source leaves (Fig. 3 and Table 2). Among the plant species

Table 2

Cell-to-cell movement of GFP, GFP/CP, GFP/TGBp1, GFP/TGBp1m12, and GFP/TGBp1m13

Proportion of sites containing GFP activity in multiple cell clusters ^a					
Plants	GFP	GFP/CP	GFP/TGBp1 ^b	GFP/TGBp1m12	GFP/TGBp1m13
<i>N. tabacum</i>	3.3% (3/90)abc	2.3% (2/88)bc	22.2% (20/90)abA	9.9% (20/202)bb	3.0% (6/201)bc
<i>N. benthamiana</i>	3.3 (3/90)ad	21.8 (31/142)ab	33.6 (36/107)aA	18.6 (18/97)abc	11.1 (10/90)ac
<i>N. clevelandii</i>	6.8 (6/88)abc	1.1 (1/90)bbc	21.0 (20/95)abA	8.3 (9/109)bb	2.2 (2/90)bbc
<i>L. esculentum</i>	2.3 (2/88)ab	3.3 (3/90)bb	14.4 (13/90)ba	3.4 (3/89)bb	3.3 (3/90)bb

^a Percentages of fluorescent cell clusters observed at 1 dpb in source leaves are indicated. Multiple-cell clusters are defined as two or more adjacent cells exhibiting GFP fluorescence. The total numbers of fluorescent sites that are multiple-cell clusters relative to the total number of fluorescent sites are in parentheses. Upper and lowercase letters indicate the results of statistical analyses. Values followed by the same lowercase letter within each column are not significantly different using Fisher's exact test at $P > 0.05$. Values followed by the same uppercase letter in each row are also not significantly different using Fisher's exact test at $P > 0.05$.

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

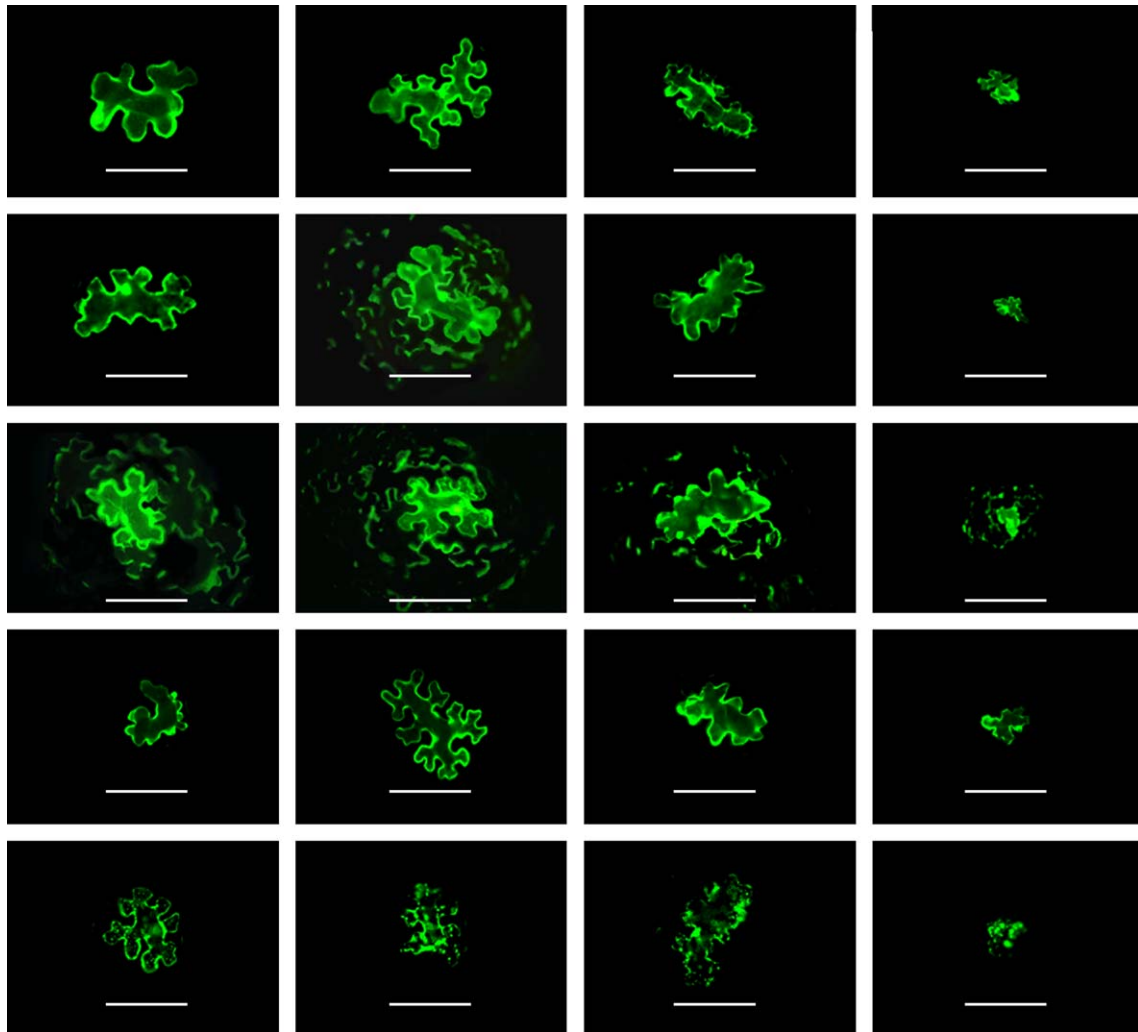


Fig. 3. Representative images of leaf epidermal cells containing fluorescence due to GFP, GFP/TGBp1, or GFP/CP. Plasmids expressing GFP, GFP/TGBp1, or GFP/CP were biolistically delivered to *N. benthamiana*, *N. tabacum*, *N. clevelandii*, or *L. esculentum* leaves. Plasmids used in each bombardment are indicated on the left and plant species for each column of images are indicated on top. Images were taken using a 20 \times objective lens. Scale bars = 20 μ m.

tested, the proportions of sites containing GFP/TGBp1 in multiple-cell clusters were significantly greater than the proportions of sites that were cell clusters containing GFP ($P < 0.05$). In *Nicotiana* spp., between 21% and 34% of sites viewed were clusters of two to five adjacent cells (Table 2), and in *L. esculentum* leaves, approximately 14% of sites were multiple-cell clusters (two to five adjacent cells). GFP/TGBp1 movement in tomato was less frequent than in tobacco species (Table 2). Statistical comparisons made among plant species determined that the proportions of cell clusters in *N. benthamiana* and *L. esculentum* represented extreme values that were significantly different (Table 2; $P < 0.05$). The proportions of cell clusters in *N. tabacum* and *N. clevelandii* were between the two extreme values and were not significantly different from either *L. esculentum* or *N. benthamiana* (Table 2; $P > 0.05$).

The proportions of multiple cell clusters containing GFP/TGBp1m12 and GFP/TGBp1m13 were significantly different from GFP/TGBp1 in all hosts tested, indicating that these

mutations were inhibitory (Table 2; $P < 0.05$). GFP/TGBp1m12 and GFP/TGBp1m13 were detected in multiple cell clusters in 18.6% and 11.1% of sites viewed in *N. benthamiana* leaves (Fig. 3 and Table 2). In *N. tabacum*, *N. clevelandii*, or *L. esculentum* leaves, between 2% and 10% of sites contained these proteins in adjacent cells (Fig. 3 and Table 2). The proportions of sites containing GFP, GFP/TGBp1m12, and GFP/TGBp1m13 were not significantly different in *N. tabacum*, *N. clevelandii*, and *L. esculentum* leaves (Table 2; $P > 0.05$). Only in *N. benthamiana* leaves were the proportions of sites containing GFP/TGBp1m12 or GFP/TGBp1m13 in multiple cell clusters significantly greater than those containing GFP in multiple cell clusters (Table 2; $P < 0.05$).

GFP/CP accumulated in multiple cell clusters in *N. benthamiana* leaves and primarily in single cells in *N. tabacum*, *N. clevelandii*, and *L. esculentum* leaves (Fig. 3 and Table 2). Only in *N. benthamiana* leaves were the proportion of sites containing GFP/CP in multiple-cell

clusters significantly greater than the proportion of sites containing GFP in multiple cell clusters (Table 2; $P < 0.05$). In *N. benthamiana* leaves, 22% of the fluorescent sites were multiple cell clusters whereas in *N. tabacum*, *N. clevelandii*, or *L. esculentum* leaves, between 1% and 3% of sites contained GFP/CP in adjacent cells (Table 2). The differences between GFP and GFP/CP accumulation in cell clusters in *N. tabacum*, *N. clevelandii*, and *L. esculentum* were not significantly different ($P > 0.05$). These data in total suggest that PVX TGBp1, but not PVX CP, possesses the ability to move between adjacent cells in *N. tabacum*, *N. clevelandii*, and *L. esculentum*.

The m13 mutation affects protein accumulation in plant cells

While the m12 and m13 mutations were designed to disrupt the TGBp1 NTPase active site motifs, additional experiments were conducted to determine if these mutations also had an effect on protein stability and subcellular targeting. BY-2 protoplasts were transfected with pRTL2-GFP, -GFP/TGBp1, -GFP/TGBp1m12, or -GFP/TGBp1m13 plasmids. GFP accumulation was measured in protoplasts at 18 h post-transfection using optical spectroscopy. GFP fluorescence was 5-fold greater than GFP/TGBp1 fluorescence, suggesting that fusion of TGBp1 to the C-terminus of GFP reduced protein expression (Fig. 4A). The difference between fluorescence due to GFP/TGBp1 and GFP/TGBp1m12 or GFP/TGBp1m13 was approximately 2-fold. The values obtained for GFP was significantly different from the values obtained from the wild type or mutant fusion proteins ($P < 0.05$); however, there were no significant differences between values obtained for the wild-type and mutant GFP/TGBp1 proteins ($P > 0.05$) based on statistical analyses.

Protein subcellular targeting was studied in *N. tabacum* (Fig. 4) and *N. benthamiana* (data not shown) leaf epidermal cells, and in BY-2 protoplasts (Fig. 4). Fluorescence due to GFP, GFP/TGBp1, or GFP/TGBp1m12 was dispersed throughout the cytoplasm in tobacco epidermal cells and in BY-2 protoplasts (Figs. 4B to D, and data not shown). The

pattern of fluorescence accumulation was quantified in BY-2 protoplasts. One hundred percent of the protoplasts analyzed by epifluorescence microscopy showed fluorescence due to GFP or GFP/TGBp1m12 dispersed throughout the cyto-

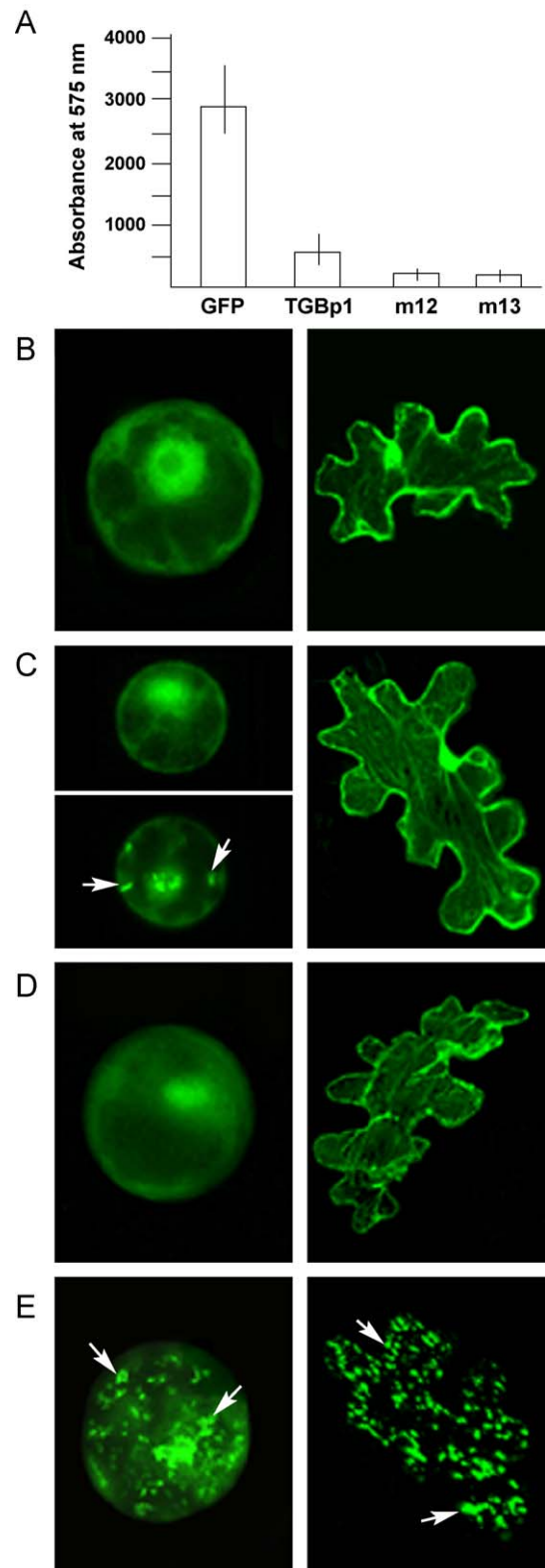


Fig. 4. Fluorometric and microscopic analysis of GFP, GFP/TGBp1, GFP/TGBp1m12, and GFP/TGBp1m13 accumulation. (A) Fluorometric data obtained from extracts derived from BY-2 protoplasts (4×10^6) transfected with pRTL2-GFP, -GFP/TGBp1, -GFP/TGBp1m12, -GFP/TGBp1m13 plasmids are presented graphically. Bars represent average absorbance values obtained in three separate experiments. Statistical analyses were conducted to compare values for GFP, wild type, and mutant GFP/TGBp1. Separate analyses were conducted comparing wild type and mutant GFP/TGBp1. Significant differences were obtained between GFP and the wild type or mutant GFP/TGBp1 proteins ($P < 0.05$) and no differences were obtained between wild type and mutant GFP/TGBp1 values ($P > 0.05$). Images of BY-2 protoplasts and *N. tabacum* epidermal cells expressing (B) GFP, (C) GFP/TGBp1, (D) GFP/TGBp1m12, and (E) GFP/TGBp1m13. White arrows in C and E indicate protein aggregates. Two different protoplasts in C represent 80% of the samples in which GFP/TGBp1 appears soluble in the cytoplasm and 20% of the samples in which fluorescent aggregates are observed.

plasm (Figs. 4B and D). Eighty percent of protoplasts expressing GFP/TGBp1 had fluorescence dispersed throughout the cytoplasm while 20% of protoplasts showed a few fluorescent aggregates (Fig. 4C). Because TGBp1 accumulates in inclusion bodies during virus infection (Davies et al., 1993), we assume that these aggregates are GFP/TGBp1-containing inclusion bodies.

Numerous fluorescent aggregates were observed in *N. tabacum* and *N. benthamiana* (data not shown) leaf epidermal cells, and in BY-2 protoplasts expressing GFP/TGBp1m13 (Fig. 4E). Ninety-three percent of protoplasts showed GFP/TGBp1m13 primarily in aggregates (Fig. 4E) and only 7% showed fluorescence throughout the cytoplasm. The abundance of these GFP/TGBp1m13-containing aggregates suggests that the m13 mutation altered protein subcellular targeting.

GFP/TGBp1 supports virus movement

To address the observed host-specific effects of CP and TGBp1 mutations in a relevant biological context, we performed experiments to examine virus movement in inoculated leaves and whole plants. Plasmids expressing GFP/TGBp1 and PVX.GUSΔ25 were co-bombarded to tobacco leaves. PVX.GUSΔ25 has 357 nt deleted from the TGBp1 coding sequence and is movement defective (Verchot et al., 1998; Yang et al., 2000) (Fig. 2). PVX.GUSΔ25 also contains the β-glucuronidase (GUS) ORF adjacent to a duplicated CP subgenomic promoter (Chapman et al., 1992). GUS histochemical staining produces a blue precipitate in cells containing wild-type PVX.GUS or PVX.GUSΔ25 viruses. GUS staining is used to monitor the spread of virus infection.

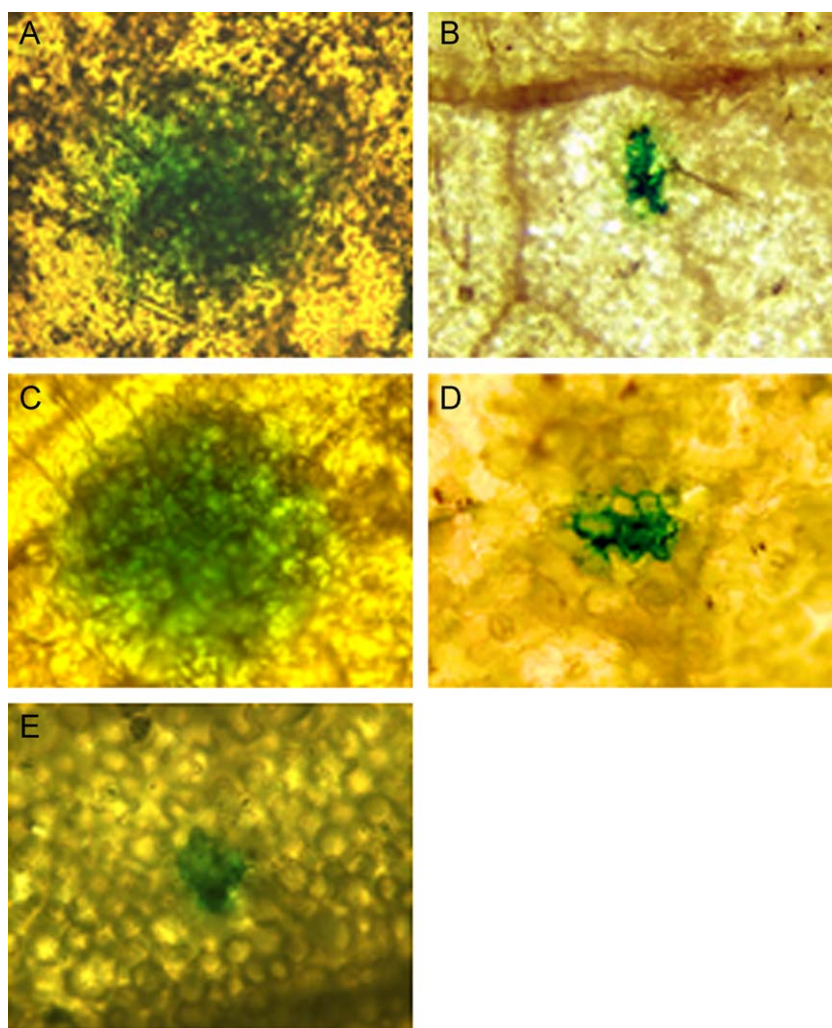


Fig. 5. Complementation of PVX.GUSΔ25 with GFP/TGBp1. Images show GUS activity in *N. benthamiana* leaves inoculated or bombarded with (A) PVX.GUS, (B) PVX.GUSΔ25, (C) PVX.GUSΔ25 plus pRTL2-GFP/TGBp1, (D) PVX.GUSΔ25 plus pRTL2-GFP/TGBp1m12, and (E) PVX.GUSΔ25 plus pRTL2-GFP:TGBp1m13. GUS histochemical staining shows an expanding infection foci in leaves 5 dpi with (A) PVX.GUS. Images in panels B through E were taken from leaves treated with GUS substrate at 3 dpb. Expanding infection foci were observed only in leaves co-bombarded with (C) PVX.GUSΔ25 plus pRTL2-GFP/TGBp1. (B, D, and E) GUS histochemical staining reveals PVX.GUSΔ25 was restricted to single cells when it was bombarded alone or with plasmid expressing GFP/TGBp1m12 or GFP/TGBp1m13. Images were taken using either a 10× or 20× objective lens.

Table 3

Systemic accumulation of PVX TGBp1 mutants in three PVX hosts species

	Proportions of plants containing PVX in upper non-inoculated leaves		
	<i>N. tabacum</i>	<i>N. benthamiana</i>	<i>N. clevelandii</i>
PVX.GUS ^a	ND ^b	12/12	ND
PVX.GUSΔ25 ^a	ND	0/12	ND
PVX204e ^c	12/12	12/12	12/12
PVX204em12	0/12	0/12	0/12
PVX204em13	0/12	0/12	0/12

^a Upper noninoculated leaves were harvested at 12 dpi and GUS assays were conducted to verify PVX.GUS accumulation. GUS activity was not detected in upper leaves of plants inoculated with PVX.GUSΔ25.

^b ND = not determined.

^c Systemic accumulation of PVX204e was first observed at 10 dpi using a UV lamp to detect GFP expression. Plants, inoculated with PVX204em12 and PVX204em13, were maintained for 14 dpi and virus was never detected in upper non-inoculated leaves.

GUS activity was detected in single cells at 3 dpb in *N. benthamiana* leaves bombarded with PVX.GUSΔ25 (Fig. 5B). GUS and GFP activities were detected in large infection foci at 3 dpb in *N. benthamiana* leaves co-bombarded with PVX.GUSΔ25 and pRTL2-GFP/TGBp1 (Fig. 5C), demonstrating that GFP/TGBp1 was indeed functionally competent. *N. benthamiana* leaves were co-bombarded with PVX.GUSΔ25 and either pRTL2-GFP/TGBp1m12 or -GFP/TGBp1m13 plasmids and then GUS assays were conducted at 3 days post-inoculation (dpi). GUS and GFP activities were restricted to single cells (Figs. 5D and E), indicating that the m12 and m13 mutations were both deleterious.

Whole plants were also inoculated with PVX.GUS or PVX.GUSΔ25 and GUS activity was used to monitor the spread of virus infection from expanding infection foci to upper noninoculated leaves (Table 3). *N. benthamiana* leaves inoculated with PVX.GUS were harvested at 3 dpi, and blue precipitate was observed in expanding infection foci (Fig. 5A). PVX.GUS was detected in upper noninoculated leaves at 12 dpi (Table 3). Blue precipitate was detected in single cells in leaves inoculated with PVX.GUSΔ25 (Fig. 5B). Plants inoculated with PVX.GUSΔ25 were maintained between 12 and 14 dpi, and GUS activity never spread beyond the initially infected cell (Fig. 5B and Table 3).

Further experiments were conducted in which the m12 and m13 mutations were directly introduced into the PVX204e infectious clone, which expresses the GFP gene from a duplicated CP subgenomic promoter (Fig. 2) (Baulcombe et al., 1995). GFP expression was used to monitor virus cell-to-cell and systemic movement. Wild-type PVX204e moved systemically in tobacco plants by 10 dpi, but PVX204em12 and PVX204em13 viruses were restricted to single cells (Table 3). Plants were maintained for 21 dpi, and there was no evidence of PVX204em12 or PVX204em13 cell-to-cell movement.

Discussion

Microinjection experiments were performed in three different host species in separate studies, and the results

support the hypothesis that TGBp1 is likely to be the primary PVX factor that can move through plasmodesmata by increasing the plasmodesmal SEL. Movement of 4.4- and 9.5-kDa F-dextran from injected mesophyll cells into neighboring cells was observed in TGB100 transgenic *N. tabacum* but not in nontransgenic *N. tabacum* leaves (Fig. 4). In two previous reports, active WCIMV or PVX TGBp1 proteins promoted movement of large 10- or 20-kDa F-dextran in *N. benthamiana* mesophyll cells and *N. clevelandii* trichome cells, respectively (Angell et al., 1996; Lough et al., 1998).

The results of the biolistic bombardment studies also indicate that TGBp1 is likely to be the primary PVX factor that can move through plasmodesmata in several plant host species. GFP/TGBp1 accumulated in multiple cell clusters in *N. benthamiana*, *N. tabacum*, *N. clevelandii*, and *L. esculentum* leaves, whereas GFP/CP was detected in multiple cell clusters only in *N. benthamiana* leaves. In other biolistic bombardment studies, GFP was fused to PVX TGBp2 and TGBp3, and GFP/TGBp2 and GFP/TGBp3 accumulated in multiple cell clusters in *N. benthamiana* leaves but were each restricted to single cells in *N. tabacum*, *N. clevelandii*, and *L. esculentum* leaves (Krishnamurthy et al., 2002, 2003). Cell-to-cell movement of GFP/CP, GFP/TGBp2, and GFP/TGBp3 in *N. benthamiana* leaves suggests that these proteins interact with an intercellular transport machinery that might not exist in other PVX host species tested.

In a previous report, it was shown that PVX TGBp1 functions to promote the cell-to-cell movement of TGBp2 and TGBp3 in *N. tabacum* leaves (Krishnamurthy et al., 2002). Following biolistic bombardment of plasmids, GFP/TGBp2 and GFP/TGBp3 each accumulated in multiple cell clusters in TGBp1-expressing transgenic leaves but were restricted primarily to single cells in nontransgenic *N. tabacum* leaves (Krishnamurthy et al., 2002).

PVX TGBp1 is related to superfamily I RNA helicases (Dolja and Carrington, 1992a; Morozov et al., 1999) and contains seven conserved amino acid sequence motifs. Motif I contains a GKS tripeptide and motif II contains a DEY tripeptide that are required for ATP hydrolysis. It has been reported that deletion or substitution mutations affecting the GKS and DEY motifs inhibited TGBp1 ATPase activity

(Donald et al., 1997; Morozov et al., 1999). The m12 mutation replaced sequences encoding the GKS tripeptide with sequences encoding an AET tripeptide. A, E, and T are similar to G, K, and S, and the substitution mutation would likely disable ATPase activity without disturbing the tertiary structure of the protein. The m13 mutation replaced sequences encoding the DEY tripeptide with sequences encoding RRF. While the m13 mutation was designed to eliminate ATPase activity by replacing D and E, which are negatively charged residues with two positively charged R residues. However, this mutation may have had an effect on the tertiary structure of the protein, as evidenced by the aggregation of GFP/TGBp1m13 seen in bombarded leaves and in protoplasts. Thus, the effects of the m13 mutation on protein subcellular accumulation were more severe than the m12 mutation. Based on these observations, we cannot conclude whether ATPase activity or protein subcellular targeting is important for TGBp1 and virus movement.

The m13 mutation is interesting because it has a general effect on protein accumulation in plant cells, which correlates with an inhibition of virus movement in *N. benthamiana* and *N. tabacum* plants. The effects of m12 and m13 mutations on protein movement in *N. benthamiana* were not as extreme as in *N. tabacum*, *N. clevelandii*, and *L. esculentum* leaves. Cell-to-cell movement of the GFP/TGBp1m12 and GFP/TG Bp1m13 is severely inhibited in *N. tabacum*, *N. clevelandii*, and *L. esculentum*, but is moderately hampered in *N. benthamiana* leaves. The results of mutational analyses provide further evidence that protein movement in *N. benthamiana* leaves might not reflect inherent properties of the proteins, but might reflect unique properties of *N. benthamiana* plants. It is possible that *N. benthamiana* plants differ from the other three PVX host species in how plasmodesmata gating is triggered, the extent to which the plasmodesmal SEL may be altered, or the cellular factors contributing to plasmodesmal transport. *N. benthamiana* leaves might either contain a positive factor that promotes protein movement through plasmodesmata (that is not present in the other host species tested) or lack a repressor that occurs in other plant species to limit protein trafficking through plasmodesmata. It is possible that there are cellular chaperones mediating protein plasmodesmal transport in *N. benthamiana* that are absent from *N. tabacum* leaves. A class of Hsp70 chaperones that contain a structural motif necessary for protein cell-to-cell transport were recently isolated from *Cucurbita maxima* plants (Aoki et al., 2002). There are also DNA-J-like chaperones that interact with some viral movement proteins (Oparka, 2004; Soellick et al., 2000). However, we do not know if these Hsp70 or DNAJ-like chaperones interact with PVX TGB proteins or CP for plasmodesmal transport. TIP is a factor isolated from *N. tabacum* leaves that interacts with TGBp2 and whose function is unknown (Fridborg et al., 2003). It is possible that TIP is a factor in *N. tabacum* that downregulates TGBp2 movement in this host. Because TIP has not been isolated from *N.*

benthamiana, we do not know if the identical factor is present or absent from *N. benthamiana*.

The least likely explanation is that the architecture of plasmodesmata in *N. benthamiana* is different than in other host species and that there is a greater opportunity for nonspecific movement of proteins between cells in *N. benthamiana* leaves. If, for example, the SEL of plasmodesmata in *N. benthamiana* is slightly larger than in other *Nicotiana* species, then the restrictions on protein cell-to-cell trafficking may be less. This explanation is not likely because GFP does not move from cell to cell in *N. benthamiana* under experimental conditions that promote cell-to-cell movement of the GFP fusion proteins, suggesting that the mechanism for protein plasmodesmata transport in *N. benthamiana* is selective.

Data obtained from studies using *N. benthamiana* have been used to build models explaining the role of the potyvirus TGB proteins in plasmodesmata transport. TGBp2, TGBp3, and CP move through plasmodesmata in *N. benthamiana* leaves, and the current model suggests that these proteins travel with virus or viral RNA through plasmodesmata (Lough et al., 1998, 2000). Because experiments conducted in other hosts fail to reproduce data obtained in *N. benthamiana* leaves, the current model likely reflects unique properties of *N. benthamiana* plants and might not explain viral protein functions in a broader context.

Materials and methods

Microinjection of mesophyll cells

Microinjection experiments were performed using previously described protocols (Ding et al., 1995; Wolf et al., 1989). Nontransgenic and TGBp1-expressing source tobacco leaves were detached and floated on 0.1 M mannitol in a petri plate. The epidermis was peeled from the abaxial side of the leaf to expose the underlying mesophyll cells. The leaf was placed on wet filter paper under the microscope with the abaxial side up. A 1-mM solution of LYCH and a 5-mM solution of either 4.4- or 9.4-kDa F-dextrans (Sigma, St. Louis, MO) were each prepared in 5 mM KHCO₃ (pH 8.0), filtered through a 0.5 µm-pore syringe filter, and stored at 4 °C (Ding et al., 1992; Fujiwara et al., 1993; Wolf et al., 1989). Mesophyll cells were injected with LYCH or FITC-dextrans using a PV820 Pneumatic Pico Pump (World Precision Instruments, Sarasota, FL) and an epifluorescence Nikon E600 microscope. Injections were carried out at an eject pressure of 5–15 psi. The PV820 has a regulated hold pressure that maintains a constant low pressure following each injection to help maintain cell turgor. The needle was withdrawn slowly to allow the injection wound to seal. The spread of fluorescence from injected cells to adjacent cells was monitored following injection and images were taken with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) attached to the microscope.

Bacterial strains and plasmids

All plasmids were used to transform *Escherichia coli* strain JM109. Plasmids pRTL2-GFP, -GFP/TGBp1, and -GFP/CP were described previously (Krishnamurthy et al., 2002; Yang et al., 2000) (Fig. 1). Two substitution mutations named m12 and m13 were introduced into pRTL2-GFP:TGBp1 and pPVX204e plasmids, respectively, replacing 9 nts encoding the GKS (GGT AAG GTC) and DEY (GAT GAG TAT) motifs with 9 nts encoding AET (GCG GAA ACC) and RRF (AGG AGA TTC) (Kalinina et al., 2002; Morozov et al., 1999). These mutations were located at positions 4585–4593 and 4729–4737 within the PVX genome. The QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) requires two mutagenesis oligonucleotides in the forward and reverse orientation. To introduce the m12 mutation into pRTL2-GFP/TGBp1 plasmids, the forward oligonucleotides, GTA GCC GGA GCC GCG GAA ACC ACA GCC CTA AGG, and the reverse oligonucleotides, CCT TAG GGC TGT GGT TTC GCG GCT CCG GCT AC, were used. To introduce the m13 mutation into plasmids, the forward oligonucleotides, TTC GCA ATC CTC AGG AGA TTC ACT TTG GAC AAC, and the reverse oligonucleotides, GTT GTC CAA AGT GAA TCT CCT GAG GAT TGC GAA, were used. Nucleotides encoding the substitution mutations are underlined.

The plasmids pPVX.GUS, pPVX.GUSΔ25K, pPVX204e, pPVX204em12, and pPVX204em13 are infectious clones of PVX with either the GUS or GFP genes inserted into the viral genome adjacent to a duplicated subgenomic promoter (Fig. 1). GUS and GFP reporters are used as a visual marker to study the spread PVX infection (Baulcombe et al., 1995; Chapman et al., 1992). The pPVX.GUS, pPVX.GUSΔ25K, and pPVX204e plasmids were prepared previously (Baulcombe et al., 1995; Chapman et al., 1992; Krishnamurthy et al., 2003; Verchot et al., 1998) (Fig. 2). The pPVX.GUSΔ25K, pPVX204e, pPVX204em12, and pPVX204em13 plasmids contain cDNA of the PVX genome adjacent to the CaMV 35S promoter (Fig. 2). The pPVX.GUS plasmids contain the PVX genomic cDNA adjacent to bacteriophage T7 promoters (Fig. 2). The pPVX.GUS.Δ25K plasmids have 357 nt deleted from the TGBp1 gene, and this mutation inhibits cell-to-cell movement of PVX (Verchot et al., 1998) (Fig. 2).

To introduce m12 and m13 mutations into the PVX genome, a fragment of pPVX204e was PCR amplified using a forward primer that anneals to the 3' end of the replicase gene (GCC AAA CAC CAC TGC ATA CCA GAG GAA ATC), as well as a reverse primer (GGC GGT CGA CAT TTA CTT GTA CAG CTC GTC CAT) that overlaps the GFP gene and contains a *SalI* (underlined) site. The PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI). Mutations were separately introduced into the pGEM-T clones using QuikChange Site-Directed Mutagenesis kit and the forward and reverse primers indicated above. Then the pPVX204e and the pGEM-T plasmids,

containing a fragment of the PVX genome encoding the m12 or m13 mutations, were digested with *AvrII* and *ApaI*. Digested pPVX204e and PVX genomic fragments containing m12 or m13 mutations were ligated.

Plant material

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

Leaves were identified as source or sink using the carboxyfluorescein (CF) dye test to ensure that the leaves used in biolistic bombardment experiments were physiologically similar. The CF dye test was developed as a means to standardize the developmental stage of the leaves being assayed (Oparka et al., 1994; Roberts et al., 1997). CF dye (Sigma) is applied to the petiole of the most mature leaf, moves through the phloem in a source-to-sink direction, and unloads in sink but not source leaves (data not shown) (Krishnamurthy et al., 2002; Oparka et al., 1994; Roberts et al., 1997; Yang et al., 2000). Source leaves were taken from similar, untreated plants for biolistic bombardment experiments.

In vitro transcription and plant inoculation

Infectious transcripts derived from pPVX.GUS plasmids were inoculated to *N. benthamiana* plants. Plasmids were linearized with *SpeI*, and in vitro transcription reactions were carried out by mixing the following: 5.0 μg of linearized DNA, 5 μl of 10× T7 transcription buffer, 2.0 μl SUPERase · Inhibitor (20 U/μl) (Ambion, Austin, TX), a 10× A/C/U/G mixture containing 20 mM ATP, CTP, and UTP, and 2 mM GTP (Pharmacia-Pfizer, Mississauga, Ontario, Canada), 5.0 μl m⁷(5')ppp(5')G Cap Analog (Ambion), 4.0 μl of T7 polymerase (Ambion), and nuclease-free water to a final volume of 50 μl. The reactions were incubated at 37 °C for 15 min before the addition of 5.0 μl 20 mM GTP (Pharmacia-Pfizer) and 1 h at 37 °C following the addition of GTP. Transcripts (10 μl) were mechanically inoculated to each leaf of an *N. benthamiana* plant and two leaves per plant were inoculated in each experiment. Experiments were repeated four times and a total of 12 plants were inoculated per construct.

Biolistic bombardment

Source leaves were detached from plants of similar age and bombarded with plasmids using the PDS 1000/He System (Bio-Rad Laboratories, Hercules, CA), as described previously (Krishnamurthy et al., 2002, 2003; Yang et al., 2000). Leaves were bombarded with 20 μg

pPVX.GUSΔ25K alone or 20 μg pPVX.GUSΔ25K combined with 20 μg of either pRTL2-GFP/TGBp1, -GFP/TGBp1m12, and -GFP/TGBp1 m13 mixed with 1 mg of 1 μm gold particles. Bombarded leaves were then stored in the dark on Phytagar (Gibco BRL, Life Technologies, Inc. Grand Island, NY) for 72 h before GUS histochemical staining (see below). In other experiments, leaves were bombarded with 10–20 μg of the pRTL2 constructs mixed with 1 mg of 1 μm gold particles. Ten microliters of a DNA/gold mixture was loaded on a carrier disk and bombarded to detached leaves as described previously (Yang et al., 2000). GFP expression was monitored in leaves bombarded with pRTL2 constructs using epifluorescence microscopy at 1 dpb.

Assays for GUS and GFP expression

PVX.GUS and PVX.GUSΔ25 inoculated leaves were vacuum infiltrated as described previously with a working solution of 10 mM EDTA, 0.5 mM K₃(FeCN)₆, 0.5 mM K₄(FeCN)₆, and 1.2 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide cyclohexylammonium salt (X-Gluc) (BD Biosciences Clontech, Palo Alto, CA), and incubated overnight at 37 °C (Dolja et al., 1992b; Verchot et al., 1998). The infiltrated leaves were preserved with 95% ethanol, and images were captured using the Optronics Magnafire camera (Intelligent Imaging Innovations, Inc., Denver, CO) attached to the Nikon E600 microscope (Krishnamurthy et al., 2002).

Confocal microscopy was conducted to collect images of GFP, GFP/TGBp1, GFP/TGBp1m12, and GFP/TGBp1m13 in bombarded leaves. The Leica TCS SP2 system was attached to a Leica DMRE microscope (Leica Microsystems, Bannockburn, IL). GFP expression was also monitored in bombarded leaves and protoplasts using a Nikon E600 (Nikon Corp., Tokyo, Japan) 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 (Krishnamurthy et al., 2002).

Plants inoculated with PVX204e, PVX204em12, or PVX204em13 viruses were monitored at 7, 14, and 21 dpi using a hand-held model B-100 BLAK-RAY long wave ultraviolet (UV) lamp (Ultraviolet Products, Upland, CA). The data were recorded with a Sony Digital Still Camera model DSC-F717 (Sony Corporation of America, New York, NY). All images taken with the Optronics or Sony cameras were edited using Adobe Photoshop version 4.0 software (Adobe Systems Inc., San Jose, CA).

BY-2 protoplast preparation and electroporation

Suspension cells of tobacco BY-2 (Nagata et al., 1992) were cultured and maintained as described in Qi and Ding

(2002) with little modification. BY-2 cells were propagated in the BY-2 culture media [Murashige and Skoog medium (MS salts; Sigma) supplemented with 30 g/l sucrose, 256 mg/l KH₂PO₄, 100 mg/l myo-inositol, 1 mg/l thiamine, and 0.2 mg/l 2,4-D, pH 5.5] on a rotary shaker at 120 rpm at 28 °C in the dark and weekly subcultured by transferring 10 ml BY-2 cells to 50 ml fresh media.

Protoplasts were isolated from the suspension cells as previously described with slight modifications (Gaire et al., 1999; Qi and Ding, 2002). Three-day-old cells in suspension culture media were collected by centrifugation at 200g for 5 min and resuspended in 1.5% cellulose “Onozuka RS” (Yakult Pharmaceutical Ind. Co., Ltd., Tokyo, Japan) and 0.2% macerace (Calbiochem-Novabiochem Corp. La Jolla, CA) in solution 1 [0.5 M mannitol, 3.6 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 5.5] and incubated for 3–5 h at 30°C in water bath with rotary shaker. The protoplasts collected by filtration through 41-μm nylon mesh (Spectrum Laboratories, Inc., Rancho Dominguez, CA) were centrifuged and washed twice with solution 1 at 100g for 5 min. Finally, protoplasts were resuspended in solution 2 (solution 1 plus 0.1 mM CaCl₂) to a density about 2 × 10⁶ protoplasts/ml and incubated on ice for 1 h.

Protoplasts were electroporated as described by Gaire et al. (1999) with a few modifications. Protoplasts (1 × 10⁶ in 0.5 ml) were mixed with 70–80 μg of plasmid DNA and 40 μg of sonicated salmon sperm DNA, as carrier. The protoplast DNA mixture was placed in a 0.4-cm gap cuvette (Bio-Rad Laboratories) on ice and then electroporated using a Gene Pulser (Bio-Rad Laboratories) at 0.18 KV, 100 Ω, and 125 μF with three pulses. Protoplasts were transferred after electroporation into a new tube containing 1 ml of solution 2, incubated on ice for 30 min, and then collected by centrifugation at 59g for 5 min. Protoplasts were resuspended in 1.5 ml of solution 3 (BY-2 culture media plus 0.45 M mannitol) and added to 6-well cell culture plates (Corning Inc., Corning, NY) containing solution 3 plus 1.0% agarose (pH 5.7). Protoplasts were cultured at 27 °C for 18 h and were then collected by centrifugation at 39g. GFP expression was assayed either by epifluorescence microscopy or fluorometric analyses (see below).

Fluorometric measurement of GFP expression in protoplasts

To measure accumulation of GFP or GFP fusion proteins in protoplasts, total protein was extracted from protoplasts (about 4 × 10⁶) by vortexing and sonicating in 100 μl of grinding buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM MgCl₂, and 10 mM DDT) (Pang et al., 1996). Samples were centrifuged at 3000g for 10 min and the supernatants were removed for fluorometric analyses. GFP expression was measured spectrally with excitation at 485 nm and emission at 535 nm in VICTOR2 D fluorometer (Perkin-Elmer, Boston, MA).

Statistical analyses

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

Fluorometric measurements of GFP expression (Fig. 4) were compared using analysis of variance (SAS Institute). Values were assigned to a completely randomized model with three replications and different plasmids as the treatment variable. Treatment means were separated using a Fisher's protected least significant difference (LSD).

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