

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

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Abstract

Potato virus X (PVX) TGBp1, TGBp2, TGBp3, and coat protein are required for virus cell-to-cell movement. Plasmids expressing GFP fused to TGBp2 were bombarded to leaf epidermal cells and GFP:TGBp2 moved cell to cell in *Nicotiana benthamiana* leaves but not in *Nicotiana tabacum* leaves. GFP:TGBp2 movement was observed in TGBp1-transgenic *N. tabacum*, indicating that TGBp2 requires TGBp1 to promote its movement in *N. tabacum*. In this study, GFP:TGBp2 was detected in a polygonal pattern that resembles the endoplasmic reticulum (ER) network. Amino acid sequence analysis revealed TGBp2 has two putative transmembrane domains. Two mutations separately introduced into the coding sequences encompassing the putative transmembrane domains within the GFP:TGBp2 plasmids and PVX genome, disrupted membrane binding of GFP:TGBp2, inhibited GFP:TGBp2 movement in *N. benthamiana* and TGBp1-expressing *N. tabacum*, and inhibited PVX movement. A third mutation, lying outside the transmembrane domains, had no effect on GFP:TGBp2 ER association or movement in *N. benthamiana* but inhibited GFP:TGBp2 movement in TGBp1-expressing *N. tabacum* and PVX movement in either *Nicotiana* species. Thus, ER association of TGBp2 may be required but not be sufficient for virus movement. TGBp2 likely provides an activity for PVX movement beyond ER association.

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Introduction

Potexviruses contain a block of three partially overlapping open reading frames termed the “triple gene block” (TGB) that encode proteins required for virus cell-to-cell movement. The triple gene block proteins are highly conserved among members of the genera *Potexvirus*, *Hordeivirus*, *Benyvirus*, and *Carlavirus* (Memelink et al., 1990; Morozov et al., 1987; Skryabin et al., 1988). In the case of *Potato virus X* (PVX) these three proteins are named TGBp1, TGBp2, and TGBp3 and they have molecular masses of 25, 12, and 8 kDa, respectively. The PVX coat

protein is also required for virus movement (Forster et al., 1992). It is likely that these proteins interact with each other to facilitate viral cell-to-cell movement; however, the nature of these interactions is poorly understood.

The potexvirus and hordeivirus TGBp1 proteins induce plasmodesmata gating, move from cell to cell, bind viral RNA, and have ATPase activity (Donald et al., 1997; Rouleau et al., 1994; Yang et al., 2000). A model was proposed suggesting that TGBp1 binds viral RNAs and transport them through plasmodesmata into adjacent cells (Donald et al., 1997; Lough et al., 1998, 2000). The PVX TGBp1 is also a suppressor of gene silencing (Voinnet et al., 2000). We do not know if this activity is essential for virus movement.

In potexviruses, hordeiviruses, and benyviruses, three separate subgenomic RNAs (sgRNA) are required to ex-

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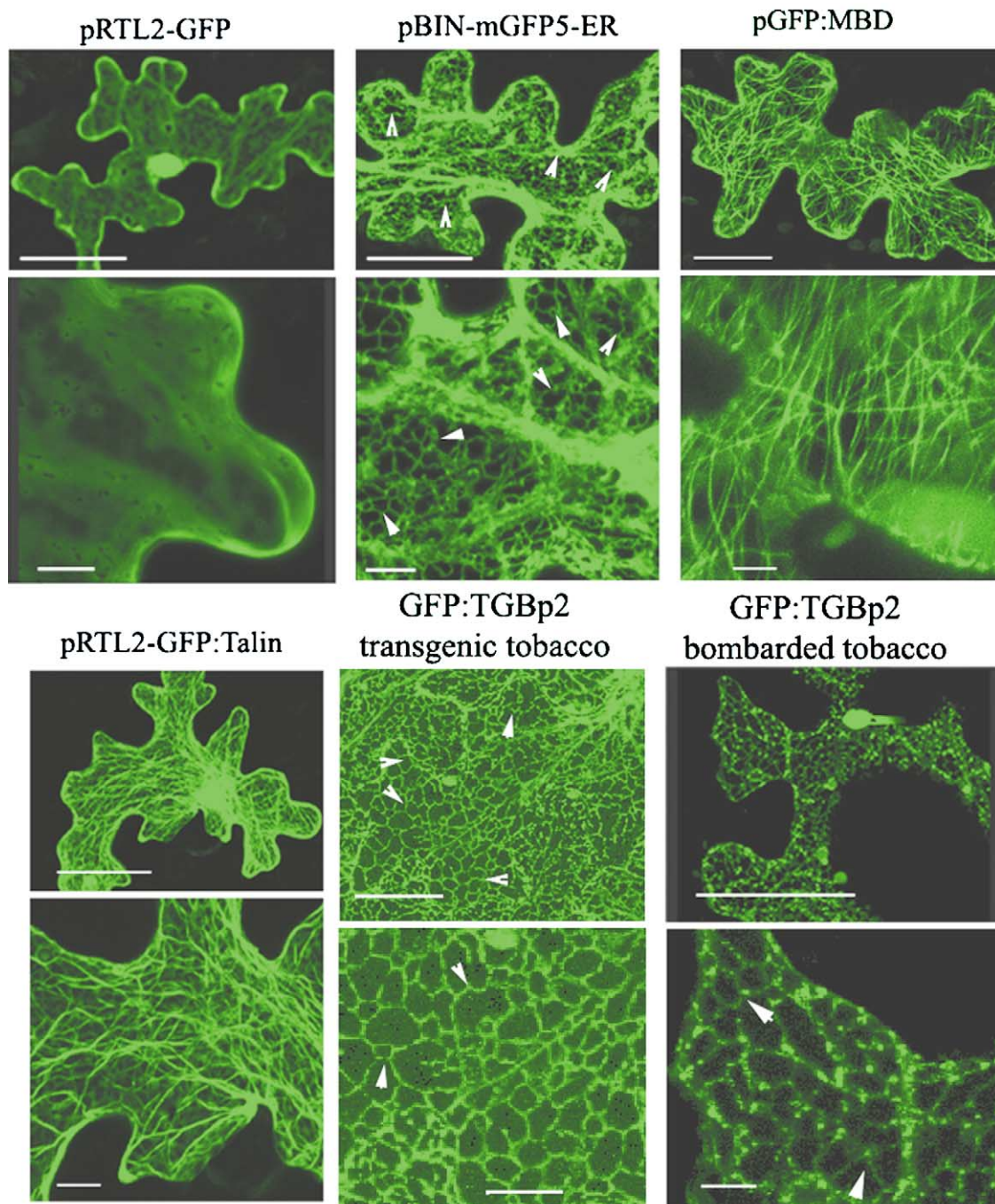


Fig. 1. Cellular localization of TGBp2, ER, microtubules, and F-actin using GFP fusions and confocal microscopy in leaf epidermal cells. Images showing fluorescence in GFP:TGBp2-expressing transgenic *N. tabacum* leaves or tobacco leaf epidermal cells transiently expressing free GFP, mGFP5-ER, GFP:MBD, or GFP:Talin. Note the association of green fluorescence with polygonal networks in tobacco expressing GFP:TGBp2 and mGFP5-ER (white arrowheads). Free GFP displays diffuse cytoplasmic fluorescence. GFP:Talin and GFP:MBD display the filamentous labeling pattern typical of the cytoskeletal network. The upper and lower panels under each heading are low and high magnification images. Bars in the upper panels represent 40 μm and in the lower panels represent 8 μm .

press the entire TGB and coat protein open reading frames (Gilmer et al., 1992; Verchot et al., 1998; Weiland and Edwards, 1994). PVX TGBp1 is expressed from a monocistronic sgRNA1, whereas TGBp2 and TGBp3 are expressed from the bicistronic sgRNA2 (Verchot et al., 1998). The viral coat protein open reading frame is expressed from

sgRNA3. In several studies of PVX (Chapman et al., 1992; Davenport et al., 1997; Kim et al., 1996) and of the related potexviruses *Foxtail mosaic virus*, *White clover mosaic virus*, and *Clover yellow mosaic virus*, only two RNA species corresponding to sgRNA1 and sgRNA3 were observed in infected plants (Bancroft et al., 1991; Bendena et al.,

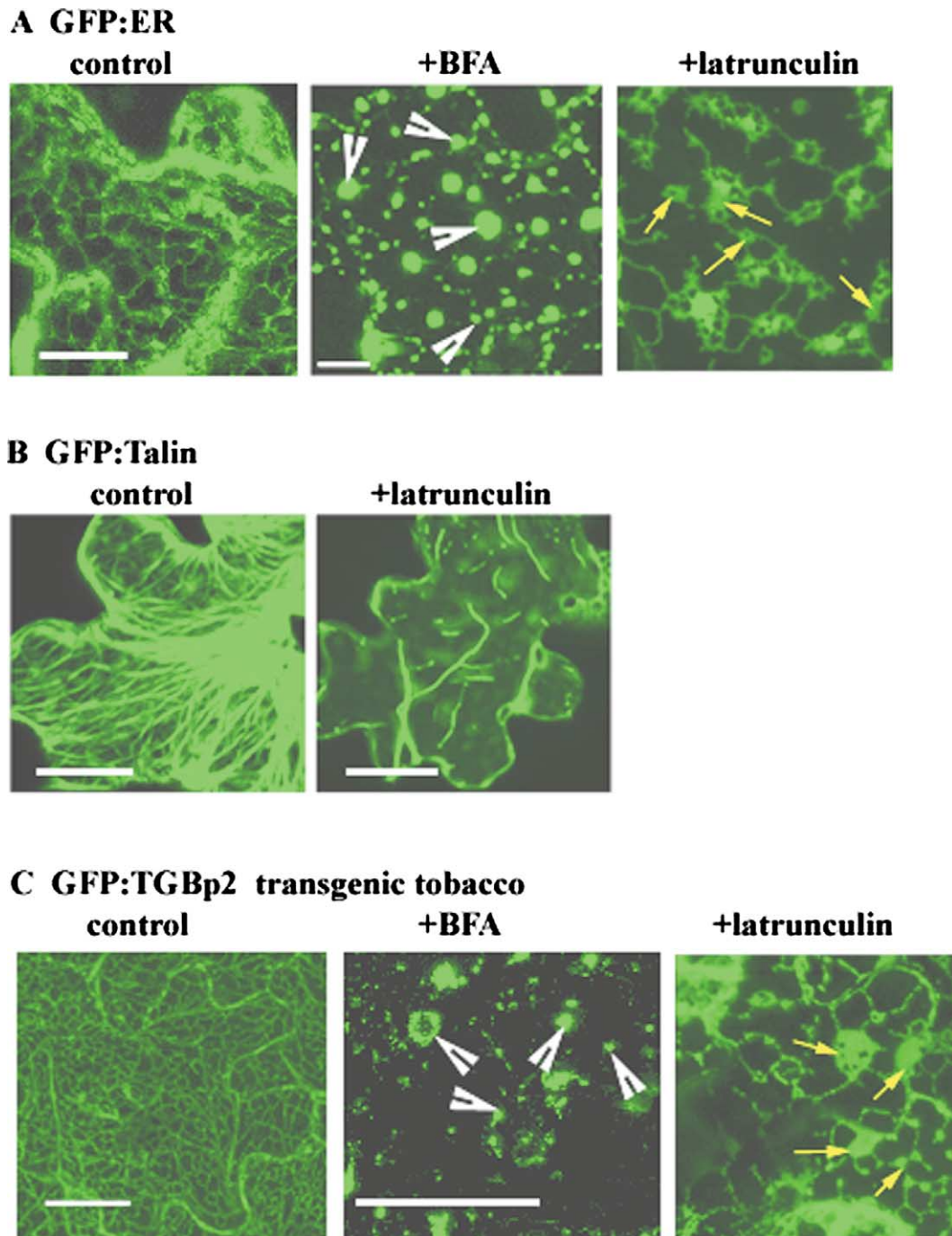


Fig. 2. Transgenic or bombarded *N. tabacum* leaves expressing various GFP fusion proteins that were treated with chemical inhibitors. Bars represent 40 μm . (A) Transgenic *N. tabacum* leaves expressing mGFP5-ER were treated with BFA or latrunculin B. The control image (untreated cell) shows the polygonal ER network. In cells treated with BFA, fluorescent aggregates (white arrowheads) that are variable in size become dispersed throughout the cell. Cisternal ER (yellow arrows) and blind-ending tubules are in latrunculin B-treated cells. (B) In leaves bombarded with pGFP:Talin, cells expressing GFP:Talin show abundant parallel actin filaments. Images show less filaments in cells treated with latrunculin B than in untreated cells. (C) Transgenic leaves expressing GFP:TGBp2 were treated with BFA, or latrunculin B. Fluorescent aggregates appeared in BFA-treated cells (white arrowheads). Cisternal ER (yellow arrows) and blind-ending tubules are in latrunculin B-treated cells.

1987; Forster et al., 1992). These data suggest that the PVX sgRNA2 accumulates to a very low level (Verchot et al., 1998).

Expression of PVX TGBp2 and TGBp3 has been difficult to study because available antibodies could not detect

these proteins in infected plants (Verchot and Baulcombe, unpublished data). Previous attempts to study TGBp2 or TGBp3 accumulation in plant cells by fusing GFP to the TGBp2 or TGBp3 open reading frames within the PVX infectious clone were unsuccessful (Verchot and Baul-

A

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GaVC  -----MSFTPPPDYN KVYLTLAIGA AVGILVHTLR SNHLTHVGDN THHLPHGGRY CDGNKRIHYN
BaMV  MDQPLHLARPPDNT RAYLVLAIGV ASALFLYTLT RNTLPHTGDN IHHLPHGGRY VDGTKGILYN
CaVX  MSGAPLRLTTPPDHT RTLFLPLTGL GLSLVVFALT RSTLPSVGDS SHSLPHGGWY RDGKTIVFYS
PVM   MPLTPPPDFDT KVYLSAALGV SLALVWLLI RSTLPVVGDR DHNLPHGGWY RDGTKSVFYN
WC1MV MPLTPPPDPQ KTYQIATLAL GLVLLAFVLI SDHSPKVGDN LHNLPHGGY KDGTKSIKYF
NMV   MPGLTTPPVNYE QVYKVLAIgf LLCASIYCLR SNHLPHVGDN IHSLPHGGNY ADGTRVQYF
PVX   MSAHGHRLTAPVNSE KVYIVLGLSF ALVSITFLLS RNSLPHVGDN IHSLPHGGAY RDGTKAILYH
CYMV  MPGLVPPPDHS KSLFVLAIGI TVVSALFVLK SHTFPIAGDN IHRFPGGGQY KDGTKQINYC
PMV   MSSHQNFLTTPPDHS KAILAVAVGV GLAIVLHFSL SYKLPSPGDN IHSLPHGGTY RDGTKSIIYN

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GaVC  GPQAGSTHVS -----SV LPFFAAITLT LVIHFICRRR R----- --VCIRCSE -----
BaMV  SP--TSSYPS -----SS LPFSMVIALA TTLFLIRNVL NPAPTPRIY APLCLHCHR -----
CaVX  GP-KKTAIN- -----WSP PIFVLEFLTLA LYVS--YLFE SRSRAG---- --SCSHCGS -----
PVM   SPGRLNSTIEA -----RKA PLLGQPWAIV VLLV--LLIW ASHKFLR--- -PNCRCACAG -----
WC1MV QRPNQHSLSK TLAKSHNT TIFLLILGLI VTLHGlyFNN NRRVSSS--- -LHCVLCQN -----
NMV   RPHSSTST-- ----HNKY TALCAVLTLS LLIFAQRLAA GNRITSV--- -SICHHCSS QGSLSGG
PVX   SPNLGSRVSL ----HNGKN AAFAAVLLLT LLIYGSYISQ RN----- ----HTCAC -----GN
CyMV  PPTHARYPKY -----PDY KWLARTAAIV IPLCLYSYHP GNNIRR---- --ICPCCNT -----Y
PMV   SPHRGPGQSG -----AL PIITVFAIIE CTLHVIKRDN PVRPQHS--- --DCPNCS- -----

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GaVC  PH-----
BaMV  NHPPC-----
CaVX  NHT-----
PVM   SHT-----
WC1MV KH-----
NMV   NHGRVSGHSELPTT
PVX   NH---SSH-----
CyMV  HHP-----
PMV   -----

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B

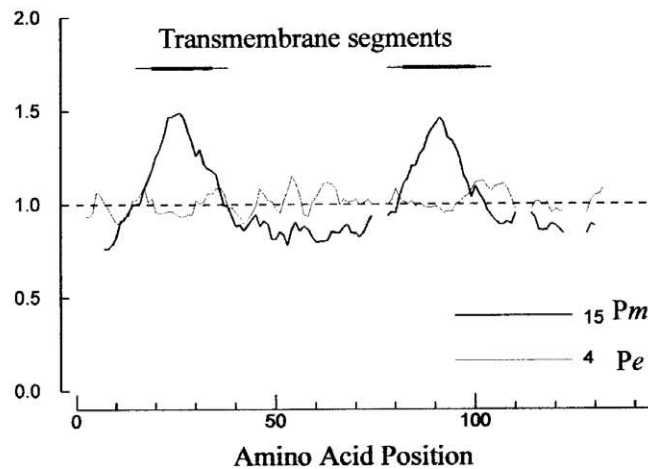


Fig. 3. Amino acid sequence analysis of potexvirus TGBp2 proteins (A) Amino acid sequence alignment was conducted using CLUSTALW and shows domains conserved among potexvirus TGBp2 proteins. Thick black bars indicate putative transmembrane domains. Gray bars indicate sites where mutations were inserted. (B) The aligned sequences were imported into TMAP program and this graphic depiction of putative transmembrane domains was obtained. The bars at the top indicate the two putative transmembrane segments. Two transmembrane domains were identified which span amino acid positions 15 to 38 and 75 to 101. The x-axis indicates the position of each residue in the TGBp2 sequence. The y-axis is the propensity value associated with each position. The black line corresponds to P_m values and values above 1.0 indicate a propensity for membrane association. TMAP uses two propensity values to predict the middle portions of the transmembrane segments (P_m) and the terminal regions of the transmembrane segments (P_e) that are based on the average scores calculated for each position in an alignment. Values above 1.0 indicate a preference to be lipid associated. Eight or more consecutive P_m values greater than 1.18 and peak values greater than 1.23 determine membrane spanning segments. P_e values above 1.08 but less than 1.17 determine termini of the membrane spanning regions and the peak P_e values were required to be 20 to 33 positions apart. Where the gray line for P_e values is above 1.0 and overlaps the black line for P_m values identifies likely ends of the transmembrane segments.

combe, unpublished data). Protein accumulation following expression from the viral genome may be too low to detect. Thus, transgenic plants expressing TGBp2, TGBp3, or the combined TGBp2/TGBp3 open reading frames were used in the past to study expression of these proteins (Verchot et al., 1998; Krishnamurthy et al., 2002). These transgenic plants complemented cell-to-cell movement of viruses containing mutations in TGBp2 or TGBp3 open reading frames and therefore served as a tool to explore protein expression and viral protein–protein interactions (Verchot et al., 1998; Krishnamurthy et al., 2002).

The green fluorescent protein (GFP) gene was fused to each PVX TGB protein coding sequence and cell-to-cell movement of GFP:TGBp1, GFP:TGBp2, and GFP:TGBp3 was studied after biolistic bombardment of plasmids to nontransgenic and transgenic *Nicotiana tabacum* expressing each of the PVX TGB or coat protein genes (Krishnamurthy et al., 2002). GFP:TGBp1 moved between adjacent cells in nontransgenic tobacco leaves but was restricted to single cells in transgenic tobacco expressing either the PVX CP or the combined TGBp2 and TGBp3 genes (Krishnamurthy et al., 2002). GFP:TGBp2 and GFP:TGBp3 moved cell-to-cell in *Nicotiana benthamiana* leaves, but did not move between cells in nontransgenic *N. tabacum* leaves (Krishnamurthy et al., 2002). GFP:TGBp2 and GFP:TGBp3 moved in source, but not sink, leaves of transgenic *N. tabacum* expressing PVX TGBp1 (Krishnamurthy et al., 2002). Thus, movement of GFP:TGBp2 and GFP:TGBp3 on the host. Moreover, in *N. tabacum* movement depends on the presence of TGBp1 as well as on the leaf developmental stage.

Amino acid sequence analyses suggested that TGBp2 and TGBp3 have hydrophobic sequences that may be transmembrane segments (Morozov et al., 1999; Solovyev et al., 2000). TGBp3 has a single transmembrane domain that binds it to the endoplasmic reticulum (ER) (Krishnamurthy et al., 2003). Mutations disrupting membrane binding of TGBp3 also disrupt virus movement, suggesting that membrane binding of TGBp3 is important (Krishnamurthy et al., 2003).

In this study, we provide evidence that PVX TGBp2 associates with the ER network in both *N. tabacum* and *N. benthamiana* leaves. Targeted mutations were introduced into computer-predicted transmembrane segments to disrupt protein-membrane associations. Effects of these mutations, on protein cell-to-cell movement and on

PVX movement were studied. Whereas the requirements for protein movement in *N. benthamiana* and *N. tabacum* differ, the requirements for virus movement may be the same in both hosts.

Results

Subcellular accumulation of GFP:TGBp2 in tobacco leaves

Two methods were used to study subcellular accumulation of PVX TGBp2 in tobacco leaves. First, transgenic *N. tabacum* were prepared that contain GFP fused with the 5' end of the PVX TGBp2 coding sequence. Second, plasmids containing GFP fused to the 5' end of the PVX TGBp2 coding sequence were biolistically delivered to tobacco leaf epidermal cells. The pattern of fluorescence was analyzed by confocal microscopy and representative examples of images obtained are shown in Fig. 1. A fluorescent polygonal network, resembling the ER network, was observed in transgenic and bombarded *N. tabacum* leaves expressing GFP:TGBp2 (Fig. 1).

The pattern of GFP:TGBp2 accumulation was compared with the pattern of mGFP5-ER, GFP:MBD, or GFP:Talin accumulation in *N. tabacum* epidermal cells. mGFP5-ER is a modified GFP containing ER-targeting and retention sequences, and therefore GFP accumulates in the ER lumen (Haseloff et al., 1997). GFP:MBD has the microtubule-binding domain of the *MAP4* gene product fused to the carboxyterminus of GFP (Marc et al., 1998), and GFP:Talin has the F-actin binding domain of the mouse *Talin* gene fused to GFP (Kost et al., 1998). In this study, stable transformants of *N. tabacum* plants expressing mGFP5-ER displayed the same reticulate network (Fig. 1) that has been described in previous studies to represent the ER network (Boevink et al., 1998; Dunoyer et al., 2002; Haseloff et al., 1997; Reichel and Beachy, 1998; Ridge et al., 1999). GFP:TGBp2 and mGFP5-ER accumulated in *N. tabacum* leaves in similar polygonal networks of thin tubules and cisternae (Fig. 1). Confocal images of cells containing GFP:MBD or GFP:Talin show filamentous arrays typical of the microtubule and actin networks, respectively (Fig. 1). The pattern of GFP:TGBp2 accumulation did not resemble the pattern of accumulation in GFP:MBD- or GFP:Talin-expressing cells.

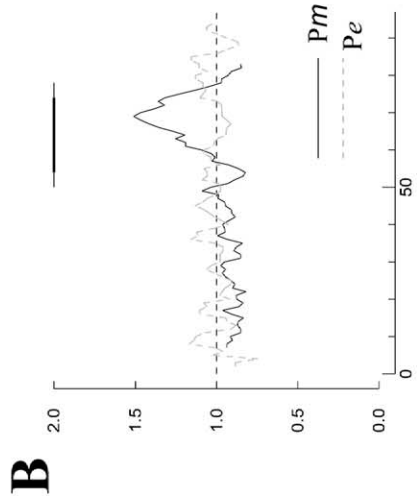
Fig. 4. Mutant pRTL2-GFP:TGBp2 plasmids bombarded to tobacco leaves. (A) Diagrammatic representation of the GFP coding sequence (gray box) fused to mutant PVX TGBp2 (open box) sequences. Thick black lines in the TGBp2 open reading frame indicate the mutations m1, m2, and m3. Changes in the TGBp2 amino acid sequence due to each mutation are indicated. (B) TGBp2 mutant sequences were imported into TMAP to determine whether the mutations should affect the predicted transmembrane domains. The graph on the left is for TGBp2m1, the middle is TGBp2m2 and the right is TGBp2m3. Bars at the top of the graphs indicate each transmembrane domain. The m1 mutation eliminates the first transmembrane domain, the m2 mutation has no effect on the transmembrane domains, and the m3 mutation eliminates the C-terminal transmembrane domain. (C) Images showing fluorescence due to GFP:TGBp2m1, GFP:TGBp2m2, or GFP:TGBp2m3 in *N. tabacum* (top panel) and *N. benthamiana* (bottom panel) leaves. GFP:TGBp2m1 and GFP:TGBp2m3 were dispersed throughout the cells in *N. tabacum* and *N. benthamiana* leaves. GFP:TGBp2m2 was in a reticulated network in *N. tabacum* and *N. benthamiana* leaves. Bars represent 40 μm .

GFP:TGBp2m1

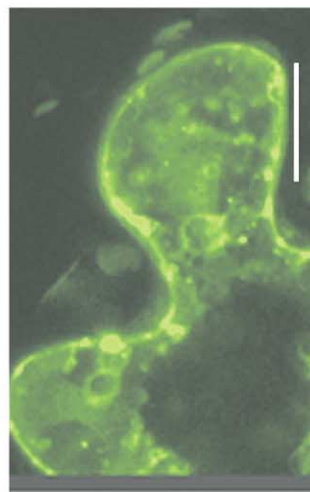
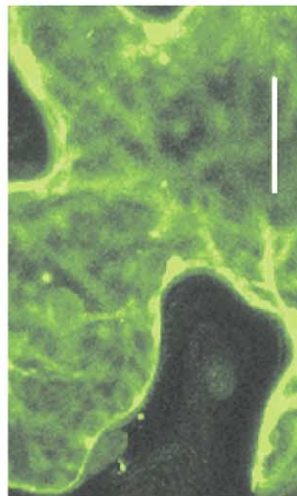
LGL-SRP substitution



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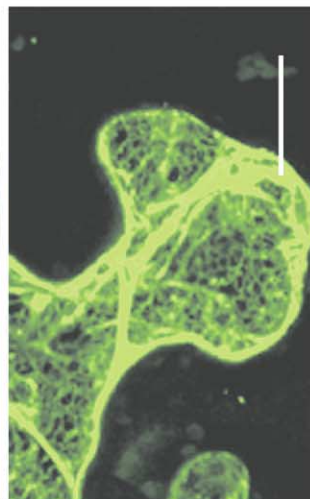
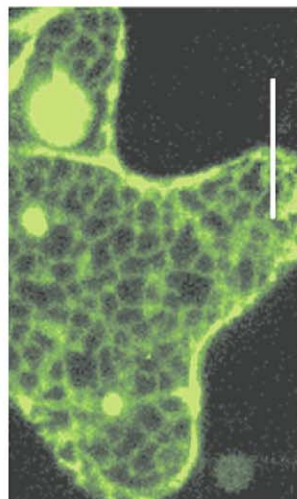
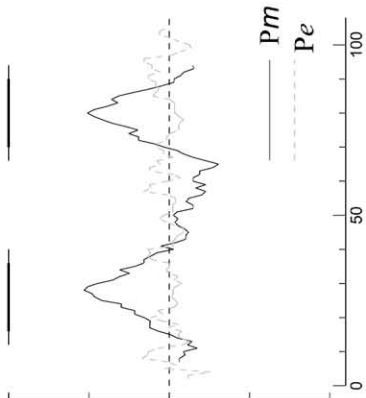
B



C

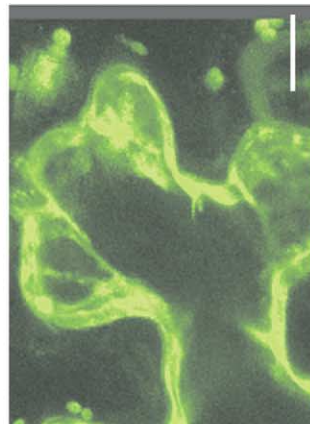
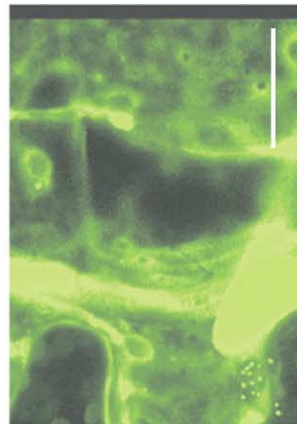
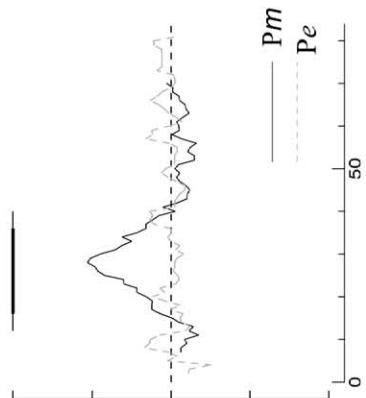
GFP:TGBp2m2

10 a.a. deletion



GFP:TGBp2m3

SRPT insertion



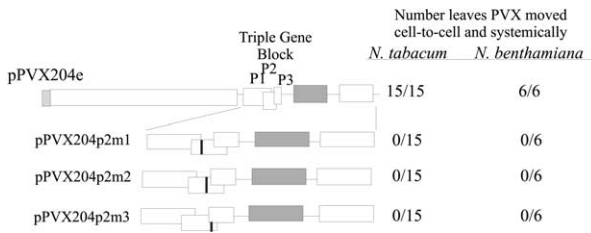


Fig. 5. The abilities of PVX204e, PVX204p2m1, PVX204p2m2, and PVX204p2m3 mutations to move cell-to-cell and systemically were compared. Diagrammatic representations of wild type and mutant PVX204e viruses are indicated on the left. Viruses were inoculated to *N. tabacum* and *N. benthamiana* plants. Total numbers of plants in which virus cell-to-cell movement relative to the total number of plants inoculated is presented. PVX204e moved cell- to-cell and systemically. None of the mutant viruses moved between adjacent cells or systemically in either *Nicotiana* species.

Chemical inhibitors that disrupt the ER network alter the pattern of GFP:TGBp2 accumulation

To test whether GFP:TGBp2 is associated with the ER or cytoskeleton, bombarded leaves were treated with brefeldin A (BFA) or latrunculin B. BFA in concentrations exceeding 100 $\mu\text{g/ml}$ severely disrupts the ER and Golgi network. BFA causes the Golgi to fuse with the ER and membranous islands form throughout the cell (Henderson et al., 1994; Ritzenthaler et al., 2002; Saint-Jore et al., 2002). If GFP:TGBp2 is ER associated, there should be islands of fluorescence scattered throughout the cell. Latrunculin B depolymerizes actin filaments (Nebenfuhr et al., 1999). Actin has been implicated in contributing to the shape and movement of the ER in plants (Knebel et al., 1990). In a previous study, chemicals that disrupt actin may also change the distribution of ER within the cell. For example, after cytochalasin D (which depolymerizes actin cables) treatment

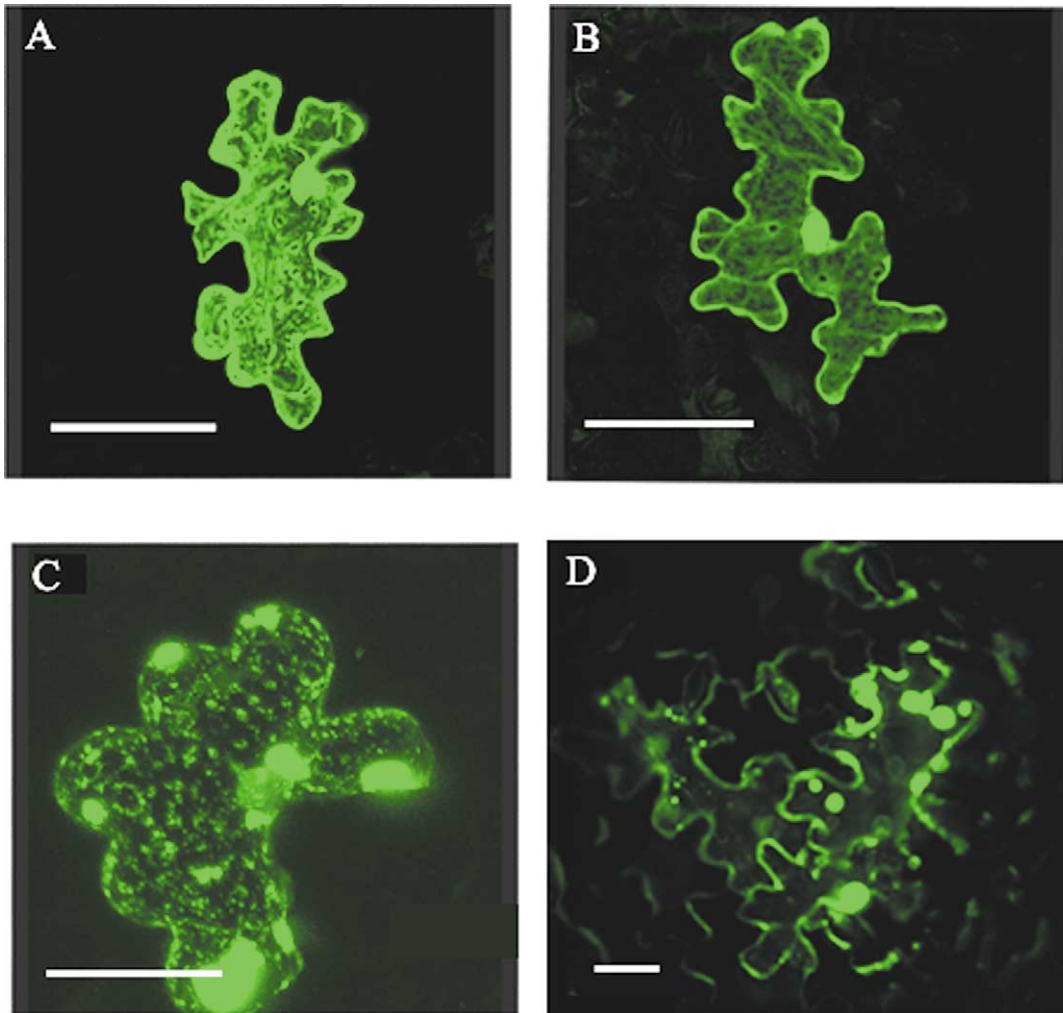


Fig. 6. Representative examples of GFP:TGBp2 accumulation in nontransgenic and TGB100 *N. tabacum* leaves following biolistic bombardment of pRTL2-GFP:TGBp2 plasmids. Multiple cell clusters recorded in Table 1 were events where GFP, GFP:TGBp2, or mutant GFP:TGBp2 proteins accumulated in two or more adjacent cells. Bars represent 100 μm . (A) Single epidermal cell of a nontransgenic *N. tabacum* leaf expressing GFP. (B) Single epidermal cell of TGB100 leaf expressing GFP. (C) Single epidermal cell of nontransgenic *N. tabacum* leaf expressing GFP:TGBp2 (D) Multiple adjacent epidermal cells of TGB100 leaf expressing GFP:TGBp2.

of cells, blind-ending ER tubules and cisternae were observed (Knebel et al., 1990). Thus, we predict that if GFP:TGBp2 is associated with the cytoskeleton, then following latrunculin B treatment we should observe blind-ending ER tubules and large cisternae.

Initially, we wanted to determine whether these drugs were generating the desired effects on the endomembrane and cytoskeleton system in *N. tabacum* leaves expressing mGFP5-ER, GFP:MBD, or GFP:Talin. A concentration of 200 $\mu\text{g/ml}$ BFA for a period of 3–4 h was necessary to cause disruption in the pattern of the ER in transgenic leaves expressing mGFP5-ER (data not shown). In contrast to the reticulate pattern consistently observed in untreated mGFP5-ER transgenic leaves, BFA-treated mGFP5-ER-expressing leaves displayed strongly fluorescent islands that were dispersed throughout the cytoplasm (Fig. 2A). In GFP:Talin-expressing cells, a 1–2 h latrunculin B treatment resulted in significantly reduced numbers of filamentous structures leaving only a few fragmented and thick actin cables in the cell (Fig. 2B). In mGFP5-ER-expressing leaves treated with latrunculin B for 1–2 h we observed blind-ending tubules and cisternae (Fig. 2A).

We then examined how these drugs affected the distribution of GFP:TGBp2. As in BFA-treated cells expressing mGFP5-ER, BFA caused extensive reorganization of the fluorescent networks in GFP:TGBp2-expressing cells (Fig. 2C). Fluorescence was in islands dispersed throughout the cell (Fig. 2C). Latrunculin B had similar effects on the reticulate network in GFP:TGBp2-expressing cells and in mGFP5-ER-expressing cells. As in mGFP5-ER-expressing cells, blind-ending tubules and sheets of cisternal ER were more often visible in GFP:TGBp2-expressing cells following treatment with latrunculin B (compare Fig. 2A and C).

PVX TGBp2 has two predicted transmembrane segments

Since the subcellular localization studies suggest that TGBp2 is associated with cellular membranes, amino acid sequence analyses were conducted to identify putative transmembrane segments of TGBp2 and mutational analysis was used to test the computer predictions. First, the PVX TGBp2 amino acid sequence was aligned with TGBp2 sequences of eight other known potexviruses using ClustalW. The alignment resembles previously published alignments containing stretches of uncharged amino acid residues and a central region of highly conserved amino acid residues (Fig. 3A) (Huisman et al., 1988; Morozov et al., 1987).

TMAP was used to identify putative transmembrane domains of the potexvirus TGBp2 proteins. TMAP is a method for predicting transmembrane helix segments from multiple aligned sequences (Persson and Argos, 1994). The prediction is based on the assumption that hydrophobic stretches of approximately 21 amino acids in length are generally required to span a lipid bilayer (Persson and Argos, 1994). The output of TMAP contains the number of predicted transmembrane segments, the predicted start and end of

each transmembrane segment, and the predicted length of each transmembrane segment (Persson and Argos, 1994). The potexvirus TGBp2 proteins have two conserved hydrophobic domains that are predicted to be transmembrane segments (Fig. 3B). The predicted transmembrane segments span amino acid position 15 to 38 and 75 to 101, within the PVX TGBp2 protein sequence. The output of TMAP predicted the N- and C-terminal regions of TGBp2, and a central domain between the putative transmembrane segments lies outside of the membrane (Fig. 3B).

To test the prediction that the two hydrophobic segments of PVX TGBp2 are transmembrane domains, three mutations (m1, m2, and m3) were introduced into the PVX TGBp2 sequence within the plasmid pRTL2-GFP:TGBp2 (Fig. 4A). The plasmid pRTL2-GFP:TbP2m1 contains a substitution mutation within the region of TGBp2 that encodes the first transmembrane segment. This mutation results in the substitution of Leu-Gly-Leu with Ser-Arg-Pro (Fig. 4A). The plasmid pRTL2-GFP:TGBp2m3 has 12 nucleotides (nts), encoding Ser-Arg-Pro-Thr, inserted into the second putative transmembrane segment. Mutations encoding Ser-Arg-Pro or Ser-Arg-Pro-Thr were specifically designed based on results of a previous report analyzing membrane insertion of the *Tobacco etch virus* (TEV) 6K membrane-spanning protein (Restrepo-Hartwig and Carrington, 1994). Nine nucleotides encoding Ser-Arg-Pro residues were introduced into the TEV 6K gene within the region encoding the transmembrane domain, and membrane insertion of TEV 6K was disrupted (Restrepo-Hartwig and Carrington, 1994). The plasmid pRTL2-GFP:TGBp2m2 has 30 nts deleted between the putative transmembrane segments (Fig. 4A). The m2 mutation is not predicted to disrupt membrane insertion of TGBp2 but may alter other TGBp2 activities (Fig. 4B). Thus only the m1 and m3 mutations are predicted to disrupt membrane insertion of GFP:TGBp2 (Fig. 4B).

The pRTL2-GFP:TGBp2m1, -GFP:TGBp2m2, and -GFP:TGBp2m3 plasmids were bombarded to nontransgenic *N. tabacum* and *N. benthamiana* leaves and intracellular protein accumulation was studied 24 h post bombardment (hpb) using confocal laser scanning microscopy. Fluorescence due to GFP:TGBp2m1 and GFP:TGBp2m3 accumulated primarily in the cytoplasm suggesting that the m1 and m3 mutations disrupted membrane insertion of these proteins (Fig. 4C). The pattern of GFP:TGBp2m2 accumulation resembled the pattern of mGFP5-ER accumulation in Fig. 1, suggesting that the m2 mutation did not alter TGBp2 membrane insertion (Fig. 4C).

TGBp2 mutations inhibited virus cell-to-cell movement

The m1, m2, and m3 mutations were each introduced into the PVX genome to determine if membrane association of TGBp2 is essential for viral cell-to-cell movement. The plasmid pPVX204e contains cDNA copies of the PVX genome adjacent to the CaMV 35S promoter. The plasmid contains the GFP gene fused to a duplicated

CP subgenomic RNA promoter inserted into the viral genome (Baulcombe et al., 1995). The plasmids pPVX204p2m1, pPVX204p2m2, and pPVX204p2m3 contain the m1, m2, and m3 mutations in the TGBp2 gene within the PVX genomic cDNA (Fig. 5). These plasmids were biolistically delivered to *N. benthamiana* or nontransgenic *N. tabacum* plants. GFP expression was used to monitor virus cell-to-cell and systemic spread.

PVX204e spread systemically within 10 days post inoculation in *N. benthamiana* or *N. tabacum* leaves. This was evidenced by GFP expression in upper noninoculated leaves in all plants bombarded with pPVX204e plasmids (Fig. 5). PVX204p2m1, PVX204p2m2, and PVX204p2m3 viruses were restricted to single cells in both *Nicotiana* species (Fig. 5). Thus, two mutations that disrupt membrane association also disrupt virus movement (in both *Nicotiana* spp). The fact that PVX204p2m2 is also defective in movement suggests that ER association is not alone sufficient to support virus movement. TGBp2 likely has additional functions beyond ER association.

TGBp2 mutations alter protein intercellular movement in N. benthamiana and TGB100 leaves

Experiments were conducted to determine if the TGBp2 mutations have an effect on cell-to-cell movement of the protein. In a previous study the pRTL2-GFP:TGBp2 plasmids were biolistically delivered to *N. benthamiana* leaves, *N. tabacum* leaves, and TGBp1-expressing transgenic *N. tabacum* leaves (named TGB100) (Krishnamurthy et al., 2002). GFP:TGBp2 moved between adjacent cells in *N. benthamiana* leaves and in TGB100 transgenic *N. tabacum* leaves, but did not move from cell to cell in *N. tabacum* leaves. Thus, in *N. tabacum* leaves, TGBp1 is essential for TGBp2 cell-to-cell movement (Krishnamurthy et al., 2002).

The plasmids pRTL2-GFP, -GFP:TGBp2, -GFP:TGBp2m1, -GFP:TGBp2m2, and -GFP:TGBp2m3 were bombarded to *N. benthamiana*, nontransgenic *N. tabacum*, or TGB100 transgenic *N. tabacum* leaves to determine if the mutations altered protein cell-to-cell movement. Cell-to-cell movement of fluorescence was monitored 24 hpb. Representative examples of protein accumulation in one or multiple cells are presented in Fig. 6.

The physiological status of these leaves was determined by CF dye translocation studies (Oparka et al., 1994; Yang et al., 2000; Krishnamurthy et al., 2002). A young leaf that imported and unloaded CF dye was determined to be a sink. A fully expanded leaf that is unable to import CF dye was a source leaf (data not shown). Following the CF dye test, leaves were detached from plants that were of a similar age and bombarded with plasmids.

GFP accumulated primarily in single epidermal cells in *N. benthamiana* or nontransgenic *N. tabacum* leaves that were bombarded with pRTL2-GFP plasmids (Table 1). On rare occasions GFP was detected in two adjacent cells. Similar background levels were reported in related studies

and might occur on occasions when plasmids were delivered to neighboring cells (Itaya et al., 1997; Krishnamurthy et al., 2002; Yang et al., 2000).

Previously, we have shown that GFP:TGBp2 accumulates in multiple cell clusters in *N. benthamiana* leaves and in transgenic TGB100 *N. tabacum*, but in nontransgenic *N. tabacum* leaves GFP:TGBp2 accumulates primarily in single cells (Krishnamurthy et al., 2002). In *N. benthamiana* source leaves in this study, 31.5% of the sites contained GFP:TGBp2 in multiple cell clusters; in nontransgenic *N. tabacum* source or sink leaves, GFP:TGBp2 accumulated primarily in single cells; and in TGB100 source leaves, 25.9% of sites contained GFP:TGBp2 in multiple cell clusters (Table 1). In TGB100 sink leaves GFP:TGBp2 was rarely detected in adjacent cells (Table 1). The proportion of cell clusters containing GFP:TGBp2 in TGB100 source leaves was significantly greater than in nontransgenic leaves ($P < 0.05$) and was significantly greater than the proportion of multiple cell clusters containing GFP in TGB100 source leaves ($P < 0.05$).

The plasmids pRTL2-GFP:TGBp2m1, m2, and m3 were each bombarded to *N. benthamiana*, nontransgenic *N. tabacum*, or TGB100 leaves to determine if these mutations alter protein cell-to-cell movement. In *N. benthamiana* leaves, the m1 and m3 mutations inhibited GFP:TGBp2 cell-to-cell movement. GFP:TGBp2m1 and GFP:TGBp2m3 were detected primarily in single epidermal cells. Approximately 8.5 and 7% of sites contained GFP:TGBp2m1 and GFP:TGBp2m3, respectively, in adjacent cells (Table 1). Statistical analyses indicate that the proportions of sites containing GFP, GFP:TGBp2m1, and GFP:TGBp2m3 in adjacent cells were not significantly different ($P > 0.05$).

The m2 mutation did not affect cell-to-cell movement of GFP:TGBp2 in *N. benthamiana*, leaves. GFP:TGBp2m2 accumulated in clusters of adjacent cells, indicating that protein cell-to-cell movement was not severely affected by this mutation (Table 1). Statistical analyses indicate that the proportions of sites containing GFP:TGBp2 and GFP:TGBp2m2 in multiple cell clusters were not significantly different ($P > 0.05$).

In nontransgenic and TGB100 source and sink leaves, GFP:TGBp2m1, GFP:TGBp2m2, and GFP:TGBp2m3 were detected primarily in single cells. Thus, each mutation introduced into the TGBp2 gene inhibited protein movement between cells in TGB100 source leaves (Table 1). Between 0 and 6% of the sites viewed in TGB100 source leaves bombarded with pRTL2-GFP:TGBp2m1, -GFP:TGBp2m2, or -GFP:TGBp2m3 contained fluorescence in two adjacent cells and these values were not significantly different from the values recorded for GFP in TGB100 leaves ($P > 0.05$) (Table 1).

These data suggest that membrane targeting of the TGBp2 protein is important for protein cell-to-cell movement in *N. benthamiana* and *N. tabacum* leaves. Whereas the m2 mutation has no effect on TGBp2 membrane insertion or on protein cell-to-cell movement in *N. benthamiana* leaves, it does alter TGBp2 intercellular movement in

TGB100 leaves. The easiest explanation is that the m2 mutation could disrupt interactions between TGBp1 and TGBp2 that are necessary for TGBp2 cell-to-cell movement. Alternatively, TGBp2 may have activities beyond ER association that contribute to its own PD transport.

Discussion

In this study, a GFP fusion with TGBp2 was detected in a polygonal pattern that resembles the ER network seen in cells transiently expressing mGFP5-ER (Fig. 1). To determine if the reticulate network was the ER network, leaf segments bombarded with pRTL2-mGFP5-ER or -GFP:TGBp2 plasmids were treated with BFA. BFA in concentrations exceeding 100 $\mu\text{g/ml}$, severely disrupts the ER network (Henderson et al., 1994; Ritzenthaler et al., 2002). In this study, BFA similarly disrupts the fluorescent network in cells containing the mGFP5-ER or GFP:TGBp2 proteins, indicating that GFP:TGBp2 likely accumulates in the ER (Fig. 2).

The ER is a dynamic network and tubules within the peripheral ER require actin to maintain their mobility. The network tubules and cisternae often change in size and position. New tubules may arise from cisternae and may elongate or retract along a trajectory. The effects of latrunculin B on the ER in mGFP5-ER- or GFP:TGBp2-expressing cells are reminiscent of the effects of cytochalasin D on the ER, as reported previously (Knebel et al., 1990). In onion bulb epidermal cells treated with cytochalasin D the ER network consisted of blind-ending tubules and large cisternae (Knebel et al., 1990). In this study, tobacco leaf epidermal cells were treated with latrunculin B and we observed blind-ending tubules and cisternal ER in mGFP5-ER- or GFP:TGBp2-expressing cells (Fig. 2A and C).

In a previous report we compared cell-to-cell movement of GFP:TGBp2, GFP:TGBp3, and GFP in *N. tabacum* leaves bombarded with plasmids using the PDS1000/He delivery system and the Helios Gene Gun delivery system (Krishnamurthy et al., 2002). GFP:TGBp2 and GFP:TGBp3 moved from cell to cell in TGB100 source leaves but not in TGB100 sink leaves or nontransgenic *N. tabacum* source or sink leaves following plasmid delivery using either system (Krishnamurthy et al., 2002). Protein movement was monitored at 1 and 3 dpb and maximal movement of GFP:TGBp2 or GFP:TGBp3 was seen at 1 dpb. Thus movement of GFP:TGBp2 or GFP:TGBp3 likely depends on intrinsic properties of the viral proteins and a specific transport mechanism (Krishnamurthy et al., 2002). GFP moved extensively between cells during a 3-day period in *N. tabacum* sink but not source leaves following plasmid delivery using the Helios Gene Gun, but was restricted primarily to single cells in leaves bombarded with the PDS1000/He system (Krishnamurthy et al., 2002). Thus movement of GFP in sink leaves may be due in part to the delivery system and in part to the developmental stage of the leaf. Other laboratories have reported occasional nonspec-

ific movement of GFP (Itaya et al., 2000; Crawford and Zambryski, 2000). Having established that nonspecific movement is reduced in studies using the PDS1000/He system this delivery system was used in this study.

Mutations in TGBp2 that disrupt ER association of TGBp2 also inhibit GFP:TGBp2 and virus cell-to-cell movement in both *Nicotiana* species. These data suggest that ER association of the protein might be important for virus and protein movement. The m2 mutation inhibited GFP:TGBp2 cell-to-cell movement only in TGB100 *N. tabacum* leaves, but inhibited PVX cell-to-cell movement in both *Nicotiana* spp. (Table 1 with Fig. 5). The fact that a single mutation, which lies outside of the transmembrane domains, inhibits protein movement in TGB100 leaves and virus movement in *N. benthamiana* and *N. tabacum* leaves suggests that TGBp2 likely has a function beyond ER membrane binding. One possibility is that TGBp2 interacts with TGBp1 in a manner that is important for virus movement. The m2 mutation might have specifically disrupted a sequence of TGBp2 that interacts TGBp1 in the TGB100 leaves. Further research is needed to determine other activities of TGBp2 and to determine if TGBp1 and TGBp2 interact.

In the current model for potexvirus cell-to-cell movement, TGBp2 and TGBp3 serve to anchor a complex of TGBp1/viral RNA/coat protein to either the ER or cytoskeletal network (Lough et al., 1998; 2000). The ER or cytoskeleton targets the “movement complex” for plasmodesmata transport. Mutations in TGBp2 and TGBp3, that disrupt membrane binding of the proteins, also disrupt virus movement (this study; Krishnamurthy et al., 2003), suggesting that ER association of TGBp2 and TGBp3 might be important for PVX cell-to-cell movement (Krishnamurthy et al., 2003). There is no evidence yet indicating a role for the cytoskeleton in potexvirus movement.

The results presented in this study and in related studies indicate that TGBp1 provides the motive force for transport of viral proteins and nucleic acids through plasmodesmata (Krishnamurthy et al., 2002, 2003). TGBp1 moves independently through plasmodesmata and does not require TGBp2 or TGBp3 to induce plasmodesmata gating or mediate its plasmodesmata transport (Yang et al., 2000; Krishnamurthy et al., 2003). TGBp2 and TGBp3 both require TGBp1 to mediate their transport in *N. tabacum* leaves.

Methods

Bacterial strains and plasmids

All plasmids were constructed using *Escherichia coli* strain JM109 (Sambrook et al., 1989). The plasmid pRTL2-GFP was obtained from Dr. B. Ding (Ohio State University, OH) and contains *EGFP* (Clontech, Palo Alto, CA). The plasmid pRTL2 contains the CaMV 35S promoter and *Tobacco etch virus* (TEV) translational enhancer element (Carrington and

Table 1
Intercellular movement of GFP, GFP:TGB2, GFP:M1, GFP:M2, and GFP:M3 in tobacco

Plants	Proportion of sites containing GFP activity in multiple cells ^a				
	pRTL2-GFP	pRTL2-GFP:TGBp2	pRTL2-GFP:TGBp2m1	pRTL2-GFP:TGBp2m2	pRTL2-GFP:TGBp2m3
<i>N. benthamiana</i>					
Source	3.2% (2/62)a	31.5% (29/92)b	8.5% (5/59)a	23.5% (20/85)b	7.0% (5/71)a
<i>N. tabacum</i>					
Source					
Nontransgenic	3.8% (15/396)a	2.3% (5/218)a	0% (0/100)a	4.5% (9/200)a	4.0% (5/126)a
TGB100	6.9 (27/389)a	25.9 (33/206)b	5.0 (6/119)a	6.0 (7/115)a	6.0 (4/67)a
Sink					
Nontransgenic	5.2 (10/191)a	5.5 (36/660)a	5.6 (22/394)a	4.0 (11/274)a	0 (0/75)a
TGB100	2.4 (20/826)a	2.7 (2/75)a	5.2 (4/77)a	5.4 (35/652)a	3.0 (5/166)a

^a Percentages of fluorescent cell clusters observed 24 hpb in source and sink leaves is indicated. The total numbers of cell clusters relative to the total number of sites containing GFP are in parentheses. Two to eight source or sink leaves of each *N. benthamiana*, *N. tabacum* nontransgenic, or *N. tabacum* TGB100 tobacco line were bombarded with each plasmid to obtain the numbers indicated. Multiple comparisons were performed for each plasmid in *N. benthamiana*, *N. tabacum* nontransgenic source, *N. tabacum* nontransgenic sink, TGB100 source, or TGB100 sink leaves. Values followed by the same letter within each row are not significantly different using PROC FREQ of SAS at $P > 0.05$. *N. tabacum* nontransgenic and TGB100 source leaves were compared for each plasmid, and values that were significantly different using PROC FREQ of SAS at $P < 0.05$ are indicated in bold.

Freed, 1990). The plasmid pBIN-mGFP5-ER plasmid was obtained from Dr. J. Hasseloff (MRC Laboratory of Molecular Biology, Cambridge, UK) and contains a modified GFP (mGFP5-ER) fused with sequences that encode ER targeting and retention signals (Siemering et al., 1996; Haseloff et al., 1997). The plasmid pGFP-MBD was a generous gift from Dr. R. Cyr (Penn State University, PA) (Marc et al., 1998). The plasmid contains GFP fused with the 5' end of the microtubule-binding domain (MBD) of *MAP4*. The plasmid pRTL2-GFP:TGBp2 contains EGFP fused with the 5' end of the PVX TGBp2 coding sequence and was described previously (Krishnamurthy et al., 2002).

All other plasmids were prepared by a two-step polymerase chain reaction (PCR) procedure (Yang et al., 2000) and sequences were confirmed using the Applied Biosystems ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (version 2.0) and an Applied Biosystems 3700 Capillary DNA Sequencer (Applied Biosystems, Foster City, CA). The plasmid pRTL2-GFP:Talin contains EGFP fused with the coding sequence of the F-actin binding domain of mouse *Talin* (Kost et al., 1998; McCann and Craig, 1997). EGFP was amplified using a forward primer containing an *XhoI* site and a reverse primer containing sequences overlapping the 3' end of the *Talin* F-actin binding domain. Similarly, the sequence encoding the F-actin binding domain of *Talin* was amplified using a forward primer complementary to the GFP reverse primer and a reverse primer containing an additional *XbaI* site. These two PCR products were annealed and the EGFP forward primer and the F-actin 3' reverse primer were used to PCR amplify the fused genes. The final PCR product was gel purified. PCR products and pRTL2 plasmids were digested with *XhoI* and *XbaI*, gel purified, and ligated.

Three mutations were separately introduced into pRTL2-GFP:TGBp2 plasmids (Krishnamurthy et al., 2002). pRTL2-GFP:TGBp2m1 has a substitution mutation located

between 60 to 68 nts from the 5' end of the TGBp2 gene. Nine nucleotides (TTAGGTCTA), which encode Leu-Gly-Leu, were replaced with 12 nts (AGTCGACCAACA), which encode Ser-Arg-Pro-Thr. The plasmid pRTL2-GFP:TGBp2m2 has 30 nts deleted between 180 and 209 nts from the 5' end of the PVX TGBp2 coding sequence. pRTL2-GFP:TGBp2m3 contains 9 nts (AGTCGACCA) inserted into the TGBp2 sequence adjacent to nt position 167 within the TGBp2 coding sequence. The 9 nts encode Ser-Arg-Pro residues.

To prepare the plasmids pRTL2-GFP:TGBp2m1, -GFP:TGBp2m2, and -GFP:TGBp2m3, complementary forward and reverse M1, M2, and M3 primers were used. The M1 primers correspond to the TGBp2 coding sequence surrounding nt positions 60 to 68 and encode a nucleotide substitution mutation. The M2 primers surround nt positions 153 to 180 within the TGBp2 coding sequence. The M2 primers contain 14 nts 5' of the deleted region and 23 nts 3' of the deleted region. The M3 primers correspond to TGBp2 coding sequences surrounding nt positions 146 to 167 plus the nucleotide insertion mutation. A similar PCR strategy was used for preparing each GFP:TGBp2 mutant. In the first PCR step, a EGFP forward primer (which contains an *NcoI* site adjacent to the EGFP translation start site) and M1, M2, or M3 reverse primers were used to PCR amplify a fragment of GFP:TGBp2 using pRTL2-GFP:TGBp2 as a template. The TGBp2 M1, M2, or M3 forward primers combined with the TGBp2 3' reverse primers were used to PCR amplify a fragment of the TGBp2 coding sequence. The 3' TGBp2 primer contains a sequence adjacent to the TGBp2 translational stop codon that was a *BamHI* restriction site. In the final step, the EGFP and the TGBp2 PCR products were annealed to each other and the EGFP forward primer and the TGB2 3' reverse primer were used to PCR amplify the GFP:TGBp2 fused genes. The GFP:TGBp2m1, GFP:TGBp2m2, or GFP:TGBp2m3 PCR products and pRTL2

plasmids were each digested with *Nco*I and *Bam*HI and then ligated.

Four PVX constructs were also prepared using two-step PCR (Verchot et al., 1998). The pPVX204 plasmid was obtained from Dr. D.C. Baulcombe (Sainsbury Laboratory, Colney, UK) and contains the PVX genome and the GFP gene. To prepare pPVX204e the original GFP was replaced with EGFP. First the TGB1 forward primer (extending from nt positions 4938–4964) and a TGB3 reverse primer (containing nts 5651–5671 of the PVX204 genome plus 15 nts of the EGFP sequence) were used to PCR amplify a fragment of the PVX genome. Then the TGB3 forward primer (overlapping nt position 5657–5671 plus 21 nts of the EGFP sequence) and the EGFP reverse primer containing an additional *Sal*I site was used to PCR amplify EGFP. The two PCR products were annealed and the TGB1 5' and EGFP 3' primers were used to PCR a large fragment. The pPVX204 plasmid and PCR products were digested with *Apa*I and *Sal*I, gel purified, and then ligated.

The pPVX204p2m1, pPVX204p2m2, and pPVX204p2m3 plasmids were also prepared using a two-step PCR procedure (Yang et al., 2000). The TGBp1 5' primer and M1, M2, or M3 reverse primers were used to PCR amplify a fragment of pPVX204e. M1, M2, or M3 forward primers and the EGFP reverse primer were used to PCR amplify a second fragment of PVX204e. These PCR products were annealed. The TGB1 forward primer and EGFP reverse primer were used to PCR amplify a final product containing M1, M2, or M3 mutations. The PCR products and PVX204 were digested with *Apa*I and *Sal*I, gel purified, and then ligated.

Plant material

N. benthamiana leaves and nontransgenic and TGB100 transgenic *N. tabacum* (cv. Petit Havana) leaves were used in these experiments. TGB100 transgenic tobacco express PVX TGBp1, are susceptible to PVX infection, and can complement cell-to-cell movement of TGBp1-defective PVX viruses, as previously described (Verchot et al., 1995, 1998).

Transgenic *N. tabacum* expressing GFP:TGBp2 was prepared using *Agrobacterium* transformation, as previously described (Verchot et al., 1995). The plasmid pRTL2-GFP:TGBp2 was digested with *Hind*III and the expression cassette that contains the CaMV 35S promoter, terminator, and the GFP:TGBp2 fused genes was transferred to the binary vector pGA482 (An, 1987). The recombinant plasmid was introduced into *A. tumefaciens* LBA4404 by triparental mating. Transgenic plants were regenerated following the leaf-disk transformation method (Horsch et al., 1985).

Biolistic bombardment

N. benthamiana source leaves, nontransgenic *N. tabacum* source and sink leaves, and TGB100 source and sink leaves were used for these experiments. Source and sink leaves

were identified by applying carboxyfluorescein dye (CF) (Sigma, St. Louis, MO) to the petiole of the most mature leaf of an *N. benthamiana* or *N. tabacum* plant (Krishnamurthy et al., 2003; Yang et al., 2000). Plants were kept in the dark overnight and the next day leaves were detached and observed using an epifluorescence microscope and a 10X objective lens. CF dye was detected uniformly in sink leaves. CF dye was detected only in the veins in source leaves. Following the CF dye experiments, source or sink leaves were detached from plants of similar age and bombarded with plasmids using the PDS 1000/He System (Bio-Rad, Hercules, CA).

Leaves were bombarded with 10 μ g plasmids mixed with 1 mg of 1 μ m gold particles. Ten microliters of a DNA/gold mixture were loaded on a carrier disk and bombarded to detached leaves as described previously (Yang et al., 2000). The leaves were observed 24 hpb using epifluorescence microscopy to detect GFP expression (Yang et al., 2000).

For plant inoculations, young *N. tabacum* or *N. benthamiana* plants were bombarded with wild-type and mutant PVX204e plasmids using the Helios gene gun (Bio-Rad, LaJolla, CA). Gene delivery cartridges were loaded with a mixture of 0.5 mg of gold particles and 5 μ g of plasmid using a Tubing Prep Station according to the manufacturer's instructions (Bio-Rad, La Jolla, CA). Gold-coated tubing was cut into 0.5 inch pieces to make the gene delivery cartridges and the used for bombardment of leaves at a pressure of 160 kPa.

Chemical inhibitor treatments of leaves

Nontransgenic *N. tabacum* or TGB100 leaves were bombarded with pBIN-mGFP5-ER, pRTL2-GFP: TGBp2, -GFP: TGBp2m1, -GFP:TGBp2m2, or -GFP:TGBp2m3 plasmids and incubated overnight (16 h) on moist filter paper.

Leaves were treated with either BFA, which disrupts the ER and Golgi network, or latrunculin B, which disrupts actin filaments (Henderson et al., 1994; Nebenfuhr et al., 1999; Ritzenthaler et al., 2002; Saint-Jore et al., 2002). Leaves were cut into segments, analyzed by epifluorescence microscopy to detect GFP expression and then transferred to either a solution of 200 μ g/ml BFA (Molecular Probes, Eugene, OR) or 1 μ m latrunculin B (A.G. Scientific, Inc., San Diego, CA). Each chemical inhibitor was dissolved in 0.1% DMSO (w/v) solution. Leaf segments were incubated in each chemical inhibitor solution for 2–4 h and then analyzed by confocal laser scanning microscopy. As a control, leaf segments were incubated in sterile water or 0.1% DMSO (w/v) for 4 h and then analyzed by confocal laser scanning microscopy.

Microscopy

GFP cell-to-cell movement in tobacco leaves was studied using a Nikon E600 (Nikon Inc., Dallas, TX) 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., Denver, CO) attached to the Nikon E600 microscope. A Leica TCS SP2 (Leica Microsystems, Bannockburn, IL) or a Bio-Rad 1024ES (Bio-Rad, Hercules, CA) confocal imaging system was used to study subcellular localization of GFP fusion proteins in tobacco epidermal cells. The Leica TCS SP2 system was attached to a Leica DMRE microscope. The Bio-Rad 1024ES system was attached to a Zeiss Axioskop (Carl Zeiss, Thornwood, NY). Both microscopes were equipped with epifluorescence and water immersion objectives. The 488-nm excitation wavelength produced by Krypton/Argon lasers was used to examine GFP expression. Fifteen to 30 optical sections were taken of each cell at 0.3- to 2- μ m intervals. All images were processed using Adobe Photoshop version 4.0 software (Adobe Systems Inc., San Jose, CA).

Statistical analyses

All data analyses were conducted using procedures from SAS (Cary, NC), and a significance level of 0.05 was used for all mean comparisons. Statistical analysis in Table 1, comparing the effects of plasmid and leaves, were assessed by chi-square tests using PROC FREQ. Each factor's simple effects were analyzed by fixing the other factors.

Sequence alignments and prediction of transmembrane domains

The TGBp2 sequences of nine potexviruses were obtained by searching the National Center for Biotechnology Information (NCBI) database using Entrez-Protein. The TGBp2 amino acid sequences of *Potato virus M* (PVM) (gi/9626092/ref/NP_056769.1), *Narcissus mosaic virus* (NMV) (gi/9626478/ref/NP_040780.1), *Bamboo mosaic virus* (BaMV) (gi/2407621/gb/AAB70564.1), *Papaya mosaic virus* (PMV) (gi/9629170/ref/NP_044332.1), *Cymbidium mosaic virus* (CyMV) (gi/2460067/gb/AAB71855.1), *Garlic virus C* (GVC) (gi/2826159/dbj/BAA61820.1), *Cactus virus X* (CVX) (gi/14602402/ref/NP_148782.1), *White clover mosaic virus M* (WCIMVM) (gi/141129/sp/P09500/VMEM), and *PVX* (gi/222445/dbj/BAA00251.1) were aligned using ClustalW (SDSC Biology Workbench, San Diego, CA). The default matrix settings (gonnet) and gap penalty (10) were maintained for this alignment. The multiple sequence alignment was imported into the TMAP version 3.2 (SDSC Biology Workbench). TMAP utilizes an algorithm to identify transmembrane segments of aligned sequences (Persson and Argos, 1994).

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