The hydrophobic segment of Potato virus X TGBp3 is a major determinant of the protein intracellular trafficking

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INTRODUCTION

Cell-to-cell movement of Potato virus X (PVX, the type member of the genus Potexvirus, family Flexiviridae) requires the viral coat protein (CP) and three movement proteins (MPs) encoded by a so-called ‘triple gene block’ (TGB), a conserved module of three partially overlapping genes (Morozov et al., 1989; Chapman et al., 1992; Lough et al., 1998, 2000; Batten et al., 2003; Adams et al., 2004; Koenig et al., 2004; Verchot-Lubicz, 2005). The TGB MPs are referred to as TGBp1, TGBp2 and TGBp3 (Solovyev et al., 1996; Morozov & Solovyev, 1999, 2003).

In general, cell-to-cell movement through plasmodesmata and long-distance transport of viral/viroid RNAs and some endogenous RNAs in plants is believed to occur in the form of specific ribonucleoprotein complexes (RNPs) (Carrington et al., 1996; Tzifra et al., 2000; Gómez & Pallás, 2004; Lucas & Lee, 2004; Opara, 2004; Waignmann et al., 2004; Heinlein & Epel, 2004). In potexviruses, transport-competent RNPs contain TGBp1, the largest TGB protein, and the viral CP (Lough et al., 1998, 2000). PVX TGBp1 is able to modify plasmodesmata and move between cells (Angell et al., 1996; Lough et al., 1998, 2000; Yang et al., 2000; Krishnamurthy et al., 2002; Howard et al., 2004; Verchot-Lubicz, 2005). Various evidence suggests that potexviral transport-competent RNPs could represent virions modified by TGBp1 (Santa Cruz et al., 1998a; Atabekov et al., 2000). TGBp1 associates in vitro with one end of filamentous virions, inducing their structural re-modelling (Atabekov et al., 2000; Rodionova et al., 2003). Accordingly, TGBp1 contains an NTPase/helicase domain (Koonin & Dolja, 1993; Morozov & Solovyev, 2003) and demonstrates NTPase, RNA helicase and RNA-binding activities in vitro (Kalina et al., 1996, 2001, 2002; Lough et al., 1998; Morozov et al., 1999; Wung et al., 1999; Hsu et al., 2004).

In rod-shaped viruses, TGBp2 and TGBp3 move to plasmodesmata and act in concert to transport TGBp1 to and through plasmodesmata (Erhardt et al., 2000; Lawrence & Jackson, 2001; Gorshkova et al., 2003; Zamyatnin et al., 2004; Haupt et al., 2005), suggesting a role in intracellular delivery of transport-competent RNPs carrying viral genomes. Recent studies have indicated that TGBp2 and TGBp3 may have additional movement-related functions (Morozov & Solovyev, 2003). In particular, potexivirus TGBp2, the
most conserved TGB protein (Koenig et al., 2004), affects the protein-trafficking capacity of plasmodesmata and facilitates movement of green fluorescent protein (GFP) between adjacent epidermal cells (Tamai & Meshi, 2001; Lucas & Lee, 2004). This activity may be a result of association with TIP, a host protein regulator of β-1,3-glucanase, which is the key enzyme of callose turnover (Fridborg et al., 2003). However, recent evidence suggests that the TGBp2 interaction with the plasmodesmata gating mechanism can be different in various plant hosts (Krishnamurthy et al., 2002; Mitra et al., 2003; Verchot-Lubicz, 2005).

PVX TGBp2 and TGBp3 exhibit properties of integral membrane proteins (Morozov et al., 1987, 1990; Krishnamurthy et al., 2003). Similar properties have been revealed for the TGBp2 and TGBp3 of rod-shaped viruses (Morozov & Solovyev, 2003). In these viruses, TGBp3, which has two transmembrane domains, is localized to peripheral endoplasmic reticulum (ER)-related membrane bodies adjacent to the plasma membrane and directs TGBp2 and TGBp1 (in the presence of TGBp2) to peripheral bodies (Solovyev et al., 2000; Zamyatnin et al., 2002; Cowan et al., 2002; Gorshkova et al., 2003; Haupt et al., 2005). The functional relevance of the TGBp3-containing peripheral bodies has been confirmed recently by observations showing that mutants of Potato mop-top virus (PMTV, genus Potomovirus) TGBp3 that are unable to form peripheral bodies are also deficient in directing TGBp1 to and through plasmodesmata (Zamyatnin et al., 2004). However, formation of peripheral bodies is not sufficient for cell-to-cell movement. For example, heterologous TGBp2 and TGBp3 directed PMTV TGBp1 to peripheral bodies; however, TGBp1 was unable to be transported through plasmodesmata (Zamyatnin et al., 2004). Additionally, cell-to-cell movement of Beet necrotic yellow vein virus was severely inhibited by overexpression of heterologous TGBp3, despite the presence of authentic wild-type TGBp3 (Lauber et al., 2005).

Molecular signals responsible for TGBp3 trafficking to peripheral bodies are yet to be elucidated. Sequence analyses of TGBp2 and TGBp3 in both filamentous and rod-shaped viruses (Morozov & Solovyev, 2003) failed to identify canonical signals of subcellular sorting (reviewed by van Vliet et al., 2003). On the other hand, in rod-shaped Potyvirus (PSLV, genus Hordeivirus), signals responsible for TGBp3 localization to peripheral bodies have been mapped to the central hydrophilic protein region conserved among TGBp3 of rod-shaped viruses and the second C-terminal transmembrane domain (Solovyev et al., 2000). Both these structural elements are absent from PVX TGBp3. Indeed, potyviral TGBp3 proteins contain a single N-terminal transmembrane segment and a characteristic conserved hydrophilic sequence, which is not similar to that of rod-shaped viruses (Morozov et al., 1991; Morozov & Solovyev, 2003).

In this study, the influence of C-terminal deletions in PVX TGBp3 on virus cell-to-cell movement was examined and protein functionality and subcellular distribution were analysed in transient expression assays. Our data confirm that PVX TGBp3 acts intracellularly and does not move from infected cells to neighbouring ones. It has also been demonstrated that fusion of GFP directly to the N-terminal hydrophobic region abrogates TGBp3 activities in intracellular trafficking and complementation tests. However, TGBp3 C-terminal truncation mutants retain the ability of wild-type protein to complement (at least partially) PVX cell-to-cell movement and to direct the intracellular movement of GFP-TGBp3. Interestingly, intracellular transport of TGBp3 from sites of its synthesis in the rough ER to the cell periphery involves a non-conventional protein trafficking pathway.

**METHODS**

**Construction of recombinant clones.** The recombinant constructs pPVX-GFP, pPVX-GFP-Xho, pPVX-GFP-Bsp, pRT-GFP, pRT-PVX.CP, pRT-PVX.25K, pRT-GFP-12K, pRT-8K and pRT-TMV-30K were described previously (Solovyev et al., 2000; Fedorkin et al., 2000, 2001).

To obtain ST-YFP, a region of rat α-2,6-sialyl transferase gene encoding the transmembrane domain and short cytoplasmic tail (52 aa) was amplified by RT-PCR of rat liver RNA (minus-sense primer 5′-GACCCCATGGCCACTTTCTCCTGGCTCTTGGC-3′ and plus-sense primer 5′-CGCCCTCGAGATGATTCATACCAACTTGAAGAAAAAGTTCAGCC-3′) and fused to the 5′ terminus of the yellow fluorescent protein (YFP) gene (Clontech Laboratories).

To obtain pRT-GFP-8K, the PVX TGBp3 gene was amplified with specific primers 5′-GGGGGATCCGGAATATCCCATGCTTCTCTG-3′ and 5′-GGGCTCTAGATCAAGGAAATCCCTGAACTG-3′ and cloned into similarly digested pRT-GFP-15K (Solovyev et al., 2000).

To construct pRT-12K/8K, pRT-12K/8KA24, pRT-12K/8KA28 and pRT-12K/8KA39, the corresponding 12K/8K gene sequences were amplified with specific primers. The forward primer 5′-CTCGAGATGTCGCCGGCAGG-3′, common for these constructs, carried an XhoI site upstream of the 12K gene sequence. The reverse primers, 5′-GACCCCATGGCCACTTTCTCCTGGCTCTTGGC-3′ for pRT-12K, 5′-GGATCCTCAATGGAAACTTAACCGG-3′ for pRT-12K/8KA24, 5′-GGCCCTCGAGATGATTCATACCAACTTGAAGAAAAAGTTCAGCC-3′ and 5′-GGGCTCTAGATCAAGGAAATCCCTGAACTG-3′ for pRT-12K/8KA28 and 5′-GGATCCTCAAGGAAATCCCTGAACTG-3′ for pRT-12K/8KA39, carried a BamHI site followed (for deletion mutants) by a stop codon downstream of the truncated 8K gene sequence. After cloning into pGEM-T (Promega), the amplified sequence was excised with XhoI and BamHI and inserted into similarly digested pRT-GFP-15K (Töper et al., 1988).

To replace the initiator codon of the PVX TGBp3 gene with ACG, a subcloned TGBp2/TGBp3-containing region of the PVX genome was amplified as two products, one obtained with an upstream primer and the specific primer 5′-GTATTTCGAACCGTAGATCAGCAAA-3′ and another with a downstream primer and the specific primer 5′-ATGTCGGTAAACTATC1TAACCGCAGT-3′. The specific primers introduced the initiator codon mutation and contained the AsuII restriction site. After digestion with Apal-AsuII and AsuII-Nhel, both amplification products were cloned into pPVX.GFP to give pPVX.GFP.AAU8SK.

pPVX.15S (kindly provided by D. C. Baulcombe, Sainsbury Laboratory, Colney, UK) represented a full-length cDNA copy of the.
PVX genome containing a TGBp2 gene mutation (an insertion of four bases, AGCT, after nt 5251 of the PVX genome) that formed a translation terminator in the TGBp2 gene sequence. To obtain pPVX.GFP.15S, the region encoding TGBp2 and TGBp3 was excised from pPVX.15S and cloned into pPVX.GFP (Fedorkin et al., 2000) to replace the wild-type sequence.

Plant material. Nicotiana benthamiana plants were grown in a greenhouse in soil (25°C, 10–12 h daylight). The largest fully expanded leaves were detached from 6-week-old plants. Such N. benthamiana leaves have been previously reported to represent transition (sink to source) leaves (Crawford & Zambryski, 2001). The level of GFP diffusion depends significantly on sink or source leaf status (Crawford & Zambryski, 2001). However, using leaves of the same age for comparative analysis allows us to avoid precise detection of this status in the cases with considerable differences in the percentage of foci showing protein egress from initially bombarded cells.

Particle bombardment and fluorescent microscopy. Particle bombardment of detached N. benthamiana leaves was performed using the flying-disk method with a high-pressure helium-based PDS-1000 apparatus (Bio-Rad) as described by Morozov et al. (1997). GFP fluorescence was detected with a Zeiss Axioscope 20 fluorescence microscope (excitation filter BP 450–490; chromatic beam splitter FT 510 and band-pass filter HQ 535/50x) or Leica TCS SP2 confocal laser scanning imaging system with excitation light of 488 nm produced by an argon laser.

RESULTS

Complementation of PVX TGBp3 deletion mutants in trans

It has been reported that some PVX carrying deletion variants of PVX TGBp3 were still capable of limited local movement, but unable to spread through the phloem (Santa Cruz et al., 1998b; Tamai & Meshi, 2001; Krishnamurthy et al., 2002). To get an insight into the role of distinct PVX TGBp3 regions in protein activity, an expression vector (pRT-12K/8K) was constructed and used for deletion mutagenesis. pRT-12K/8K carried the overlapping genes of TGBp2 and TGBp3 under the control of the Cauliflower mosaic virus (CaMV) 35S RNA promoter (Fig. 1). The pRT-12K/8K-transcribed mRNA, which mimics a viral subgenomic RNA for expression of TGBp2 and TGBp3, could serve as a template for translation of both proteins and maintain the low level of TGBp3 that is characteristic of TGB expression during virus infections (Dolja et al., 1987; Zhou & Jackson, 1996; Verchot et al., 1998; Johnson et al., 2003). pRT-12K/8K was modified to produce constructs pRT-12K/8K, pRT-12K/8K and pRT-12K/8K, which carried the genes of C-terminally truncated TGBp3 mutants that lacked 24, 28 and 39 C-terminal amino acid residues, respectively (Fig. 1). The conserved TGBp3 signature CxGxG (Morozov & Solovyev, 2003) was unaffected in pRT-12K/8K, partially deleted in pRT-12K/8K and fully removed in pRT-12K/8K (Fig. 1).

To analyse functional competence of the TGBp3 C-terminally truncated mutants in virus cell-to-cell movement, we tested their ability to complement transport of pPVX.GFP.ΔAUG8K, the GFP-tagged PVX infectious cDNA clone in which the AUG initiation codon of the TGBp3 gene was converted into ACG (Fig. 1). This mutation did not affect the encoded amino acid sequence of the overlapping TGBp2 gene. Microprojectile bombardment of pPVX.GFP.ΔAUG8K onto fully expanded N. benthamiana leaves and subsequent fluorescent microscopy at 3 and 4 days post-infection (p.i.) revealed that the mutant caused infection that was mainly confined to single cells (Fig. 2). In control experiments, pPVX.GFP.15S, a pPVX.GFP derivative with a frame-shift mutation in the TGBp2 gene (Fig. 1), gave rise to foci consisting mostly of single fluorescent cells (Fig. 2), whereas mean sizes of GFP-containing foci produced by pPVX.GFP at these time points were 20±4 and 44±6 cells, respectively (data not shown; Fedorkin et al., 2000, 2001).

In the control co-bombardment of pPVX.GFP.ΔAUG8K with pRT-12K/8K, more than 80% of infection foci consisted of three or more cells and the mean focus size was 5±15 cells, showing that the bicistronic 12K/8K construct was able to complement PVX.GFP.ΔAUG8K as efficiently as the monocistronic 8K construct, which provided a higher expression level of PVX TGBp3 (Fig. 2). Importantly, in these co-bombardment experiments, almost all secondarily infected cells were connected to the bombarded cells and the mean size of infection foci remained nearly unchanged between 3 and 4 days p.i. (Fig. 2; data not shown). In contrast to TGBp3 complementation, co-bombardment of movement-deficient TGBp1 or CP PVX mutants with vectors expressing the respective functional genes resulted in infection foci of more than 12 cells at

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**Fig. 1.** Mutations in PVX TGBp2 and TGBp3. Shaded boxes represent the coding sequences of TGBp2 and TGBp3. Dark grey boxes indicate the hydrophobic sequence segments. The position of conserved sequences in TGBp3 is shown. Angles indicate deletions. Boxes with a dotted outline indicate untranslated sequences that are either downstream of an artificially introduced terminator codon (mutant 15S) or lack the initiator AUG codon due to replacement by ACG (mutant 12K/ΔAUG8K).
In co-bombardment with PVX.GFP.DAUG8K, the three C-terminally truncated TGBp3 mutants demonstrated a similar level of movement complementation. Compared with the foci produced by the movement-deficient PVX.GFP.DAUG8K, the number of foci consisting of one cell was decreased in co-expression experiments from 83.0 to 23.4–28.3 %, whereas the percentage of foci with three or more infected cells increased from 12.0 to 46.9–57.1 % (Fig. 2). Accordingly, complementation resulted in an increase in the mean focus size (Fig. 2). These data show that even complete deletion of the conserved hydrophilic region of PVX TGBp3 (mutant 8KΔ39) did not block the ability of mutant to complement cell-to-cell transport of PVX.GFP.DAUG8K. However, the efficiency of complementation was obviously lower in comparison with the wild-type TGBp3 gene (Fig. 2). These data confirm earlier findings showing that the 8K gene mutant expressing only the N-terminal protein half is still capable of supporting a restricted virus cell-to-cell movement (Santa Cruz et al., 1998b).

Subcellular localization and lack of functional competence of PVX TGBp3 fused to GFP

For subcellular localization studies, the GFP gene was fused to the 5′ end of the PVX TGBp3 gene as described by Krishnamurthy et al. (2002) and placed under the control of the CaMV 35S RNA promoter producing the expression vector pRT-GFP-8K. Fluorescent microscopy of N. benthamiana leaves bombarded with pRT-GFP-8K revealed association of the fusion protein with ER tubules (Fig. 3a), as previously reported by Krishnamurthy et al. (2003).

Unlike GFP-fused PVX TGBp3 targeted to the ER, TGBp3 encoded by PSLV and PMTV has been found in peripheral bodies (Solovyev et al., 2000; Zamyatnin et al., 2002, 2004; Gorshkova et al., 2003). To test whether transiently expressed PVX TGBp3 is also able to move to peripheral bodies, co-bombardment of pRT-GFP-8K and full-length infectious cDNA clone pPVX201 containing the PVX genome under the control of the CaMV 35S RNA promoter (kindly provided by D. C. Baulcombe, Sainsbury Laboratory, Colney, UK) was used. Fluorescent microscopy of co-transfected epidermal cells revealed that, in this case, GFP-fused TGBp3 was localized to peripheral bodies (Fig. 3b and c), which is typical of the situation found in cells expressing PSLV GFP-TGBp3 (Solovyev et al., 2000; Gorshkova et al., 2003). To analyse further the relationship between subcellular localization of GFP-fused TGBp3 and the wild-type protein, N. benthamiana leaves were co-bombarded with pRT-8K. Fluorescent microscopy of transfected epidermal cells showed that GFP-TGBp3 was localized to peripheral bodies (Fig. 3d). These data suggest that subcellular localization of GFP-fused PVX TGBp3 differed from the distribution of non-fused PVX TGBp3 and the wild-type protein can direct GFP-TGBp3 to peripheral bodies.

Previously, wild-type PVX TGBp3 has been shown to direct...
GFP-fused TGBp2 to peripheral bodies (Solovyev et al., 2000), whereas bombardment of N. benthamiana leaves with pRT-GFP-8K alone revealed that most of the individually expressed TGBp2 of PVX were associated with numerous vesicles of an unknown nature (Fig. 3e). This type of TGBp2 distribution resembles the behaviour of GFP-12K in Nicotiana tabacum cells (Mitra et al., 2003). However, co-bombardment of pRT-GFP-12K with pRT-8K resulted in redirection of GFP-fused TGBp2 to peripheral bodies (Fig. 3f) (Solovyev et al., 2000). To test whether GFP-fused PVX TGBp3, like the wild-type protein, is able to influence the subcellular localization of PVX TGBp2 (Solovyev et al., 2000), leaves were co-bombarded with pRT-GFP-12K and pRT-GFP-8K. In transfected cells, GFP fluorescence was associated with the ER network (Fig. 3g), whereas peripheral bodies typical of co-expression of GFP-fused TGBp2 and non-fused TGBp3 (Fig. 3d) were never observed. These data demonstrate that the fused GFP

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Fig. 3. Intracellular trafficking of PVX TGBp3. (a) GFP fluorescence in N. benthamiana leaf epidermal cells bombarded with pRT-GFP-8K. (b, c) Localization of GFP-8K in a cell co-bombarded with pRT-GFP-8K and PVX infectious cDNA clone pPVX201. One cell is presented as a projection of a series of optical sections (b) and a single optical section in a median cell layer (c). (d) Subcellular localization of GFP-8K in a cell co-bombarded with pRT-GFP-8K and pRT-8K. (e) GFP fluorescence in a cell bombarded with pRT-GFP-12K. (f) Subcellular localization of GFP-12K in a cell co-bombarded with pRT-GFP-12K and pRT-8K. (g) GFP fluorescence in a cell co-bombarded with pRT-GFP-12K and pRT-GFP-8K. (h, i) Localization of GFP-8K in the presence of 8K mutants in cells co-bombarded with pRT-GFP-8K and either pRT-12K/8KΔ24 (h) or pRT-12K/8KΔ39 (i). (j, k, l) Two-colour imaging of cells co-bombarded with pRT-GFP-8K, pRT-8K and pRT-ER-YFP. The GFP signal is shown in (j); the YFP signal (k) was digitally pseudocoloured with red to facilitate interpretation of the merged image (l). All images, except (c), represent a projection of a series of optical sections. Bars, 10 μm.
molecule hindered the ability of TGBp3 to target TGBp2 to peripheral bodies.

The functional competence of GFP-8K was also tested in co-bombardment of pPVX.GFP.ΔAUG8K and pRT-GFP-8K. In this experiment, both the mean size of infection foci and the percentage of foci consisting of one cell were similar to those in pPVX.GFP.ΔAUG8K-bombarded control leaves (Fig. 2). In contrast, the non-fused 8K efficiently complemented cell-to-cell movement of pPVX.GFP.ΔAUG8K (Fig. 2). These data demonstrate that the GFP fusion of PVX TGBp3 is dysfunctional.

**PVX TGBp3-containing peripheral bodies are ER-derived membrane structures**

Since TGBp3 expression caused relocation of GFP-fused TGBp3 from the ER network, one could propose that TGBp3 directed ER disruption and its reorganization into condensed peripheral bodies. To verify this hypothesis, *N. benthamiana* leaves were co-bombarded with three expression vectors, pRT-GFP-8K, pRT-8K and pRT-ER-YFP. The last plasmid carried the gene of ER-targeted YFP (Zamyatnin et al., 2004). In bombarded cells, as expected, GFP fluorescence was detected in peripheral bodies (Fig. 3j). ER-YFP was found both in the typical ER structures and the peripheral bodies (Fig. 3k and l). Thus, expression of TGBp3 did not disrupt the ER. Importantly, the presence of the ER marker in the TGBp3-containing peripheral bodies demonstrated that they represent membrane structures of an ER origin.

**Subcellular localization of the C-terminally truncated mutants**

When a mixture of vectors pRT-GFP-8K and pRT-12K/8K was used for bombardment of *N. benthamiana* leaves, GFP fluorescence was associated with the peripheral bodies as in the case of co-bombarded vectors pRT-GFP-8K and pRT-8K (Fig. 3d; data not shown). Thus, even a suppressed level of TGBp3 expression resulted in targeting of GFP-TGBp3 to peripheral bodies.

The expression vectors pRT-12K/8KΔ24, pRT-12K/8KΔ28 and pRT-12K/8KΔ39 (Fig. 1) were individually co-bombarded with pRT-GFP-8K. For all mutants, GFP fluorescence observed 20–24 h after co-bombardment was associated with the peripheral bodies, which is similar to the situation found in co-bombardment of pRT-GFP-8K with pRT-12K/8K (Fig. 3h and i; data not shown). Thus, even complete deletion of the conserved hydrophilic region of PVX TGBp3 (mutant 12K/8KΔ39) did not block the ability of the protein to reach the peripheral sites. These data suggest that signals responsible for intracellular protein trafficking to the peripheral compartments are located in the N-terminal hydrophobic sequence of PVX TGBp3.

**TGBp3 intracellular transport to peripheral bodies is COPII-independent**

In yeasts, animals and plants, the transport of membrane proteins from the ER to destination cell compartments involves, as a rule, COPII-coated vesicles (Nebenführ, 2002; Barlowe, 2003; Bonifacino & Lippincott-Schwartz, 2003; van Vliet et al., 2003). Formation and budding of the COPII transport vesicles on the ER membranes requires a small GTPase, Sar1 (Andreeva et al., 2000; Pasqualato et al., 2002; Barlowe, 2003). To determine whether the transport of TGBp3 from the ER to peripheral membrane bodies is COPII-dependent, Sar1[T39N], the previously described dominant negative mutant of Sar1 that prevents COPII budding complex formation (Andreeva et al., 2000), was constructed.

To verify whether Sar1[T39N] had the expected effect on the COPII-dependent vesicular transport in our experimental system, a marker protein (ST-YFP) was used that represented YFP fused to the N-terminal signal anchor sequence of a rat sialytransferase as described by Saint-Jore et al. (2002). This fusion has been previously shown to localize to Golgi stacks of plant cells and to be transported from ER to Golgi via the COPII pathway (Boevink et al., 1998; Saint-Jore et al., 2002; Brandizzi et al., 2004). When expressed in *N. benthamiana* epidermal cells, ST-YFP was indeed found in Golgi stacks (Fig. 4a). Co-expression of ST-YFP with Sar1[T39N] resulted, as described previously (Andreeva et al., 2000; Nebenführ, 2002; Ritzenthaler et al., 2002), in localization of YFP fluorescence in ER elements (Fig. 4b) or an ER-like network partially converted to a system of lamellar cisterns punctured by irregularly sized holes (Fig. 4c). Presumably, these two non-mutually-exclusive phenotypes reflected different amounts of Sar1[T39N] expressed in a particular cell. When pRT-GFP-8K and pRT-12K/8K were co-expressed with Sar1[T39N] and ST-YFP, GFP fluorescence was associated with typical peripheral bodies, whereas the blockage of COPII-dependent transport in the same cell was easily detected by changes in ST-YFP localization (Fig. 4d). These data show that the dominant negative mutant of Sar1 had no effect on TGBp3 intracellular transport, which could occur, therefore, in a COPII-independent manner. Importantly, co-bombardment of pRT-GFP-8K and full-length infectious cDNA clone pPVX201 with Sar1[T39N] also resulted in Sar1-independent intracellular GFP localization to peripheral bodies (Fig. 4f).

Thus, when co-expressed with wild-type TGBp3, GFP-tagged PVX TGBp3 reached peripheral bodies in spite of blockage of the COPII-dependent ER exit by the GDP-restricted Sar1 mutant (Fig. 4d). Conversely, it was found that, in transient co-expression, Sar1[T39N] inhibited peripheral body formation by pRT-GFP-8K in the presence of pRT-12K/8KΔ24, the mutant with the smallest C-terminal deletion leaving the central conserved TGBp3 region intact (Fig. 4e). This unexpected finding strongly suggests that even the small C-terminal deletion in mutant
8KΔ24 prevents TGBp3 access to the trafficking pathway, which bypasses COPII transport.

**DISCUSSION**

In cell-to-cell movement of the PVX genome, transport-competent RNPs (non-virion RNP or virions) include TGBp1 and CP. Both these proteins have been demonstrated to move through plasmodesmata (Batten *et al.*, 2003; Morozov & Solovyev, 2003; Howard *et al.*, 2004; Verchot-Lubicz, 2005). This paper provides evidence that PVX TGBp3, in contrast, is unable to move between cells. When cell-to-cell transport of PVX.GFP.DAUG8K was transiently complemented by wild-type TGBp3, virus movement was restricted to the cells immediately adjacent to initially infected cells (Fig. 2). This is in agreement with earlier findings suggesting that potexviral TGBp3, unlike TGBp1 and CP, functions only intracellularly (Lough *et al.*, 2000; Tamai & Meshi, 2001). On the other hand, previous experiments have suggested that GFP-fused PVX TGBp3, unlike the non-fused protein, is capable of cell-to-cell movement (Krishnamurthy *et al.*, 2002, 2003), which might point to differences in properties of PVX TGBp3 and its GFP fusion. Indeed, our data showed that PVX TGBp3 with GFP fused to its N terminus was deficient in (i) its functionality in complementation of virus cell-to-cell movement, (ii)

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**Fig. 4.** Effects of Sar1[T39N] on protein subcellular localization. (a) Localization of ST-YFP. (b, c) Effect of Sar1[T39N] on localization of ST-YFP. (d) Co-expression of pRT-GFP-8K and pRT-12K/8K with ST-YFP and Sar1[T39N]. (e) Co-expression of pRT-GFP-8K and pRT-12K/8K/Δ24 with ST-YFP and Sar1[T39N]. (f) Co-expression of pRT-GFP-8K with ST-YFP and Sar1[T39N] in the presence of the cloned PVX genome pPVX201. In (d), (e) and (f), the GFP signal is shown in the left panels; the YFP signal (middle panels) was digitally pseudocoloured with red to facilitate interpretation of merged images (right panels). Pictures taken 20 h after bombardment. (a, b, c, f) Bars, 10 μm; (d, e) bars, 20 μm.
subcellular targeting and (iii) the ability to direct TGBp2 to peripheral bodies. It should be noted that this conclusion prompted us to construct another fusion gene in which the GFP gene was fused to the C terminus of PVX TGBp3. However, GFP-fused TGBp3 appeared to give no fluorescence in bombarded *N. benthamiana* cells (data not shown), possibly as a result of, for example, protein misfolding and low stability. Thus, PVX TGBp3 is a example of a protein that has become deficient in functional and localization properties as a result of fusion to the fluorescent protein (Brândizzi *et al*., 2002; Kim *et al*., 2002; Lalonde *et al*., 2003). However, similarly to the GFP-fused MP of CaMV (Thomas & Maule, 2000), this hindrance can be overcome by co-expression with wild-type TGBp3, which restores intracellular trafficking of the GFP-fused TGBp3 (Fig. 3d). Since PSLV TGBp3 is able to form protein complexes (Gorshkova *et al*., 2003), one cannot exclude that PVX TGBp3 is also capable of self-interaction. If PVX TGBp3 functions require dimer/oligomer formation, trafficking of GFP-fused TGBp3 molecules might be blocked due to their inability to form such complexes with each other, but restored by non-fused TGBp3 still able to interact with the GFP fusion.

In the presence of native TGBp3, GFP-fused TGBp3 was found in peripheral bodies that represented membrane structures containing an ER marker (Fig. 3j–l). Subcellular localization of PVX TGBp3 is similar to that of hordeiviral TGBp3, which has been previously shown to accumulate in ER-derived peripheral membrane bodies associated with plasmodesmata (Solovyev *et al*., 2000; Zamyatnin *et al*., 2002; Gorshkova *et al*., 2003). Similar peripheral bodies were also induced by PMTV TGBp3 (Zamyatnin *et al*., 2004; Haupt *et al*., 2005). These observations strongly suggest that PVX TGBp3-containing peripheral bodies represent enlarged forms of structures functionally linked to the virus translocation pathway through plasmodesmata rather than simple inclusion bodies where superexpressed protein is deposited. Therefore, in spite of possible artefacts in the experimental system, which should always be kept in mind when working with GFP-fused protein expressed at high levels, such as mistargeting and misfolding of fusions or their detrimental effect on the functioning of the whole cell due to overexpression (Brândizzi *et al*., 2002), the presented data on co-localization with the ER marker protein and observations of peripheral bodies in PVX-infected cells (Fig. 3b and c) strongly support the functional relevance of these structures.

The potexviral TGBp3 consists of two structural elements, the N-terminal hydrophobic sequence, which potentially forms a transmembrane domain, and the C-terminal hydrophilic region containing a sequence motif conserved in the TGBp3 of representatives of the *Flexiviridae* (Fig. 1) (Morozov *et al*., 1991; Adams *et al*., 2004; Morozov & Solovyev, 2003). The hydrophobic sequence is necessary for interaction with cell membranes and protein activity in cell-to-cell movement (Morozov *et al*., 1991; Krishnamurthy *et al*., 2003). Moreover, fusion of GFP to this N-terminal hydrophobic segment inhibits functional activity of PVX TGBp3 (see above). In this paper, the role of the protein hydrophilic C-terminal region in the functional competence of the protein was also analysed in complementation tests and targeting to cell peripheral compartments. For these experiments, three PVX TGBp3 mutants with C-terminal truncations of 24, 28 or 39 aa residues (8KΔ24, 8KΔ28 and 8KΔ39, respectively) were constructed (Fig. 1). Note that the N-terminal transmembrane segment was intact in these mutants. In co-expression with PVX.GFP.ΔAAUG8K, the TGBp3 mutants were functionally competent. They demonstrated a similar level of cell-to-cell movement complementation, although this was, however, lower than that of the wild-type TGBp3 (Fig. 2).

Upon co-expression in epidermal *N. benthamiana* cells, both of the two C-terminally truncated TGBp3 mutants tested directed GFP-fused TGBp3 to the peripheral bodies, similar to the situation found with wild-type TGBp3 (Fig. 3h and i). We believe that at least part of the peripheral structures observed with the C-terminally truncated mutants are identical to the bodies directed by the wild-type protein because of the ability of mutant proteins to complement PVX.GFP.ΔAAUG8K (see above). Therefore, one can conclude that these mutants have retained the ability of TGBp3 to complement cell-to-cell trafficking of movement-deficient PVX and to move to the peripheral bodies. Moreover, even complete deletion of the hydrophilic region of PVX TGBp3 (mutant 8KΔ39) did not block the ability to direct GFP-fused TGBp3 to the peripheral sites. However, some peculiarities of intracellular sorting are likely to be perturbed by deletions in the PVX TGBp3 hydrophilic region. In co-bombardment experiments, such deletion variants induced formation of large amorphous TGBp2-containing inclusions in the cell interior in addition to typical peripheral bodies (our unpublished data).

Our data suggest that indispensable signals for intracellular protein trafficking to the peripheral compartments are located in the N-terminal transmembrane hydrophobic sequence of PVX TGBp3. Similarly, translocation of hordeiviral TGBp3 to the cell periphery requires a signal in its C-terminal hydrophobic sequence (Solovyev *et al*., 2000; our unpublished data). Also, trafficking and localization of some cell membrane proteins were found to be determined by the composition and the length of their hydrophobic sequences (particularly the N-terminal hydrophobic segment) (Rayner & Pelham, 1997; Letourneur & Cosson, 1998; Reggiori *et al*., 2000; Szczesna-Skorupa & Kemp, 2001; Watson & Pessin, 2001; Dirnberger *et al*., 2002; Goder & Spiess, 2003).

Further studies are required to resolve the molecular details of the TGBp3 N-terminal transmembrane region-specified translocation pathway to peripheral bodies. As a rule, transport of membrane-anchored proteins from the sites of their synthesis in the ER involves COPII-coated transport vesicles, which bud from ER membranes in ER exit sites (ERES) distributed throughout the cell and later fuse to the
Golgi (Nebenfähr, 2002; Barlowe, 2003; van Vliet et al., 2003; daSilva et al., 2004; Hawes, 2005). Budding of the COPII vesicles requires a small GTPase, Sar1, which initiates the formation of budding membrane complexes containing cargo proteins (van Vliet et al., 2003). To get an insight into the TGBp3 trafficking pathway, a known method was used to disrupt the ER exit of proteins by co-expression with a dominant-negative GDP-restricted mutant of GTPase Sar1, Sar1[T39N], which prevents COPII prebudding complex formation (Andreeva et al., 2000; Brandizzi et al., 2004). Sar1[T39N] efficiently blocked the ER exit of ST-YFP, a control Golgi marker (Fig. 4b and c) (Neumann et al., 2003; Brandizzi et al., 2004). However, GFP-tagged PVX TGBp3 reached peripheral bodies in the presence of wild-type TGBp3 in spite of blockage of the COPII-dependent transport by Sar1[T39N] (Fig. 4d). Thus, the intracellular transport of TGBp3 to peripheral bodies involves a COPII-independent pathway. Indeed, evidence has been provided for the existence of such non-classical trafficking systems for protein transport to the plasma membrane and vacuoles (Mitsuhashi et al., 2001; Törmäkangas et al., 2001; Neumann et al., 2003; Siddiqi et al., 2003; Delmas et al., 2004; Tamura et al., 2004). Unfortunately, only very limited information is available on the molecular mechanisms that target cargo proteins to non-conventional ER export routes (Nebenfähr, 2002; Barlowe, 2003; Bonifacino & Lippincott-Schwartz, 2003). Interestingly, the ability of TGBp3 to enter the COPII-independent trafficking pathway is blocked by the short C-terminal truncation in 8K_D (Fig. 4e). So, this mutant may only enter the conventional COPII-dependent

Fig. 5. Model of TGB-mediated cell-to-cell movement. (a) Formation of membrane transport vesicles carrying TGBp2 and TGBp3. TGBp2 and TGBp3 are translated on ER-bound ribosomes (shown as pairs of grey ovals). In the Golgi-derived pathway, TGBp2/TGBp3 can reach the Golgi in Sar1-independently formed ER-derived vesicles (steps 1a and 1b) or by a Sar1-dependent transfer mechanism that does not involve vesiculation (step 1b') (daSilva et al., 2004; see text). Further TGBp2/TGBp3 transport involves sliding of Golgi stacks along actin filaments (step 1c) and formation of Golgi-derived TGBp2/TGBp3-containing vesicles. Alternatively, in a Golgi-independent pathway, TGBp2/TGBp3 are transported in ER-derived vesicles formed Sar1-independently (step 2a) and capable of movement along actin filaments (step 2b). (b) Delivery of TGB-specific vesicles to plasmodesmata-associated sites and virion/RNP movement through plasmodesmata. Driven by targeting signal(s) of TGBp3, ER-derived or Golgi-derived vesicles bound to TGBp1-containing virions or movement-competent RNPs are fused to putative TGBp3 receptor(s) (not depicted) located either on the ER tubule (step 3) or on the plasma membrane (step 4) at the plasmodesmal orifice. These events might induce virion/RNP interaction with postulated plasmodesmal receptor(s) responsible for further translocation through the plasmodesmal microchannels (step 5). These receptors may be represented by plasma membrane- or desmotubule-embedded proteins, as well as receptors in spoke-like extensions (Roberts & Oparka, 2003; Heinlein & Epel, 2004; Oparka, 2004). In the case of transport via plasma membrane receptor sites (step 4), recycling of TGBp2/TGBp3 to the ER can occur (step 6). Localization of a peripheral body on the ER in the close vicinity of the plasmodesmal entrance is indicated.
trafficking pathway from the ER. It is conceivable that the wild-type TGBp3 may use both COPII-independent and common COPII-dependent pathways.

The data presented here allow us to propose a model for the TGBp3 trafficking mechanism and TGBp3-directed movement of viral RNA and proteins. According to this model (Fig. 5), TGBp3 can accumulate in small membrane vesicles formed on the ER membrane in a Sar1-independent manner (Fig. 5a, step 1a) and then fuse to Golgi stacks (Fig. 5a, step 1b), as documented for the general pathway of membrane protein trafficking (van Vliet et al., 2003). Also, TGBp3 can reach the Golgi without formation of discrete transport vesicles via a newly discovered mechanism of Sar1-dependent sliding of ER vesicles together with individual Golgi stacks along the ER membrane (daSilva et al., 2004; Hawes, 2005) (Fig. 5a, step 1b'). In both cases, further TGBp3 transport to plasmodesmata occurs via Golgi movement along actin filaments (Fig. 5a, step 1c) and further formation of Golgi-derived vesicles (Fig. 5a, step 1d) (Hawes, 2005). Alternatively, the TGBp3 can be delivered to plasmodesmata via a Golgi-independent pathway in ER-derived transport vesicles that are probably transported to plasmodesmal orifices along actin filaments (Fig. 5a, steps 2a and 2b) (Roberts & Oparka, 2003; Oparka, 2004; Haupt et al., 2005). Whatever the origin of the TGBp3-specific transport vesicles, these carriers serve to deliver TGBp2, TGBp1 and viral RNA to plasmodesmata-associated sites using targeting signal(s) in TGBp3 (Solovyev et al., 2000; Zamyatnin et al., 2002; 2004; Gorshkova et al., 2003; Morozov & Solovyev, 2003; Oparka, 2004; Haupt et al., 2005). TGBp2 and TGBp3 enter the ER co-translationally due to integration of their hydrophobic regions into the ER membrane. Importantly, translation of TGBp2 and TGBp3 is co-ordinated in the process of reading a single subgenomic RNA (Morozov & Solovyev, 2003), which might provide a mechanism for TGBp2 and TGBp3 co-segregation for ER exit in the same transport vesicles. TGBp2/TGBp3-containing vesicles (or Golgi stacks) bind to movement-competent virions (or RNPs) through protein–protein interactions and/or by direct association with RNA (Morozov & Solovyev, 2003).

The specificity of vesicle delivery is provided by putative TGBp3 receptor(s) located in the area of the plasmodesmal cytoplasmic sleeve (Roberts & Oparka, 2003; Oparka, 2004). Interaction of these receptor(s) with TGBp3-specific transport vesicles most probably induces their fusion to either the plasma membrane (Haupt et al., 2005) or the cortical ER in the vicinity of a plasmodesmal orifice (Fig. 5b, steps 3 and 4) (Lucas, 1999; Lee et al., 2003; Oparka, 2004). Further poorly characterized steps, particularly increase of plasmodesma size exclusion limit and translocation of viral movement-competent RNPs through plasmodesmal microchannels, involve a number of interactions with postulated plasmodesmal receptors (Fig. 5b, step 5) (Lough et al., 1998; Lucas, 1999; Tamai & Meshi, 2001; Mitra et al., 2003; Zamyatnin et al., 2004; Heinlein & Epel, 2004; Oparka, 2004). In the case of delivery of a viral RNA-containing complex to receptor(s) located in the plasma membrane (Fig. 5b, step 4), TGBp2 and TGBp3 may recycle together through the endocytic pathway to the ER compartment corresponding to peripheral bodies (Fig. 5b, step 6) (Haupt et al., 2005; Murphy et al., 2005). Thus, this model emphasizes the indispensable role of TGBp3 in directing intracellular trafficking of other TGB proteins and viral RNA to plasmodesmata-associated sites. We believe that the new model provides a good basis for further experimental verification of the molecular details of TGB-mediated movement of plant viruses.

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