Journal of General Virology (2003), **84**, 1351–1366 DOI 10.1099/vir.0.18922-0

Review

Triple gene block: modular design of a multifunctional machine for plant virus movement

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Many plant virus genera encode a 'triple gene block' (TGB), a specialized evolutionarily conserved gene module involved in the cell-to-cell and long-distance movement of viruses. The TGB-based transport system exploits the co-ordinated action of three polypeptides to deliver viral genomes to plasmodesmata and to accomplish virus entry into neighbouring cells. Although data obtained on both the TGB and well-studied single protein transport systems clearly demonstrate that plant viruses employ host cell pathways for intra- and intercellular trafficking of genomic nucleic acids and proteins, there is no integral picture of the details of molecular events during TGB-mediated virus movement. Undoubtedly, understanding the molecular basis of the concerted action of TGB-encoded proteins in transporting viral genomes from cell to cell should provide new insights into the general principles of movement protein function. This review describes the structure, phylogeny and expression of TGB proteins, their roles in virus cell-to-cell movement and potential influence on host antiviral defences.

INTRODUCTION

Plant viruses require virus-encoded proteins to move from cell to cell via plasmodesmata (PD). The non-virion 30 kDa protein of *Tobacco mosaic virus* (TMV) was the first specific viral protein identified that could support intercellular plant virus spread (Leonard & Zaitlin, 1982; Ohno *et al.*, 1983; Deom *et al.*, 1987) and, therefore, was defined as a 'transport protein', or, currently, 'movement protein' (MP) (Hull, 1989; Atabekov & Taliansky, 1990). Functions that have been definitively or tentatively assigned to the 30 kDa-like MPs (Melcher, 2000) include targeting of viral RNA to PD and increase in the effective PD pore size (SEL, size exclusion limit) to allow trafficking of the RNA or an RNA–MP complex (ribonucleoprotein complex, RNP) through the pore (Carrington *et al.*, 1996; Lazarowitz & Beachy, 1999; Leisner, 1999; Lucas, 1999; Tzfira *et al.*, 2000; Blackman & Overall, 2001; Haywood *et al.*, 2002; Heinlein, 2002a).

A number of positive-stranded RNA viruses have been found to lack gene products with similarity to the TMV MP (Mushegian & Koonin, 1993). Comparisons of genomic sequences in some such viruses revealed a strikingly similar element of three partially overlapping ORFs called the 'triple gene block' (TGB) (Bouzoubaa *et al.*, 1986; Morozov *et al.*, 1987, 1989; Forster *et al.*, 1988; Huisman *et al.*, 1988; Skryabin *et al.*, 1988; Rupasov *et al.*, 1989). TGB-encoded proteins are referred to as TGBp1, TGBp2 and TGBp3, according to the positions of their genes (Solovyev *et al.*, 1996). Further accumulation of plant virus genome sequence data revealed TGBs in the genera *Potexvirus*, *Carlavirus*, *Allexivirus*, *Foveavirus*, *Hordeivirus*, *Benyvirus*, *Pomovirus* and *Pecluvirus* (Fig. 1). While arrangement of the TGB cistrons relative to each other is well conserved, TGB positions in the genomes of viruses of different genera can vary considerably (Fig. 1) (Morozov *et al.*, 1989; Morozov & Solovyev, 1999). Mutational analyses of infectious cDNA clones of virus genomes demonstrate that all three TGB proteins are essential for the virus movement process (Petty & Jackson, 1990; Petty *et al.*, 1990; Beck *et al.*, 1991; Gilmer *et al.*, 1992; Herzog *et al.*, 1998). Thus, movement functions carried on the single TMV MP are likely to be distributed over three proteins in TGB-containing viruses, a feature that makes such viruses an attractive model to investigate the movement process.

Published ahead of print on 18 March 2003 as DOI 10.1099/vir.0.18922-0

Phylogeny and sequence comparisons of TGB proteins

The TGB is found in only some viruses of the 'alpha-like' or 'Sindbis-like' supergroup (Fig. 1) (Koonin & Dolja, 1993; Mushegian & Koonin, 1993; Morozov & Solovyev, 1999), a feature that might reflect emergence of the TGB in virus(es) of this phylogenetic branch followed by co-adaptation between replication and movement genes.

TGBp1 contains a NTPase/helicase sequence domain that is closely related to the replicative helicases of alpha-like viruses and belongs to helicases of superfamily I (SF-I) (Fig. 2) (Gorbalenya *et al.*, 1989; Gorbalenya & Koonin, 1993; Koonin & Dolja, 1993). Of seven typical motifs in this

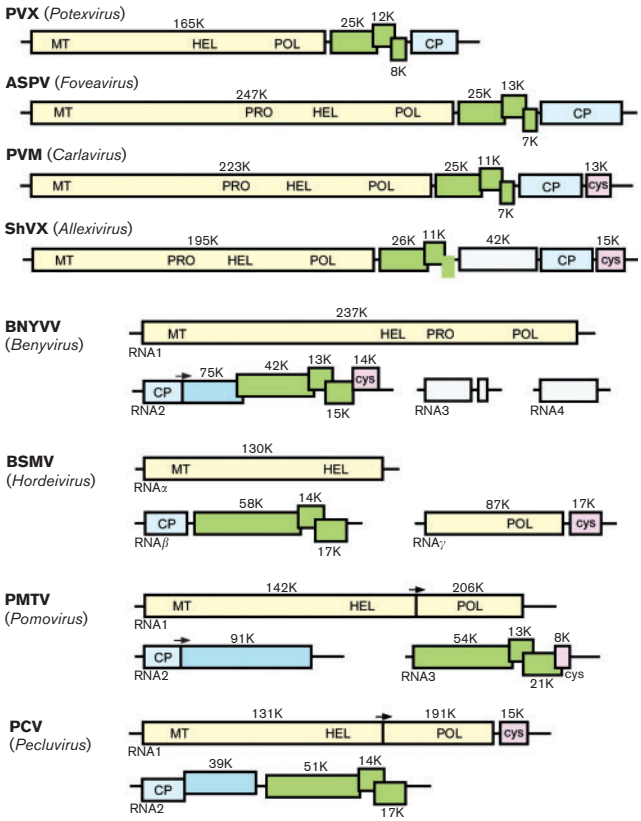

Fig. 1. TGB in viruses of different genera. Genes are shown as boxes and molecular masses of encoded proteins are indicated. TGB is shown in green. Genes of replicative proteins are shown in yellow and the locations of conserved protein sequence domains of methyltransferase (MT), protease (PRO), helicase (HEL) and polymerase (POL) are indicated. CP, coat protein gene; cys, genes of cysteine-rich proteins. Arrows depict weak terminator codons that can undergo readthrough. Readthrough domains of CPs are shown in dark blue. The third TGB gene of *Shallot virus X* (ShVX) lacks the initiator AUG codon and is indicated by an open box. PVX, *Potato virus X* (X05198); ASPV, *Apple stem pitting virus* (D21829); PVM, *Potato virus M* (X53062); ShVX, *Shallot virus X* (M97264); BNYVV, *Beet necrotic yellow vein virus* (X05147, X04197, M36894 and M36897); BSMV, *Barley stripe mosaic virus* (U35768, U35772 and U13918); PMTV, *Potato mop-top virus* (AJ238607, NC_003724 and AJ277556); PCV, *Peanut clump virus* (X78602 and L07269).

domain, motif I, with a characteristic GKS/T tripeptide, and motif II are responsible for binding ATP and Mg^{2+} and correspond to the 'Walker A' and 'Walker B' sites found in numerous ATP-binding proteins (Figs 2 and 3) (Gorbalenya & Koonin, 1993; Kadare & Haenni, 1997). Phylogenetic analysis of the NTPase/helicase sequences allows clustering of TGBp1 into two major groups, corresponding to filamentous viruses (genera *Potexvirus*, *Carlavirus*, *Foveavirus* and *Allexivirus*) and rod-shaped viruses (genera *Hordeivirus*, *Benyvirus*, *Pomovirus* and *Pecluvirus*). Furthermore, the molecular masses of TGBp1 in filamentous viruses range from 24 to 26 kDa and the NTPase/helicase domain

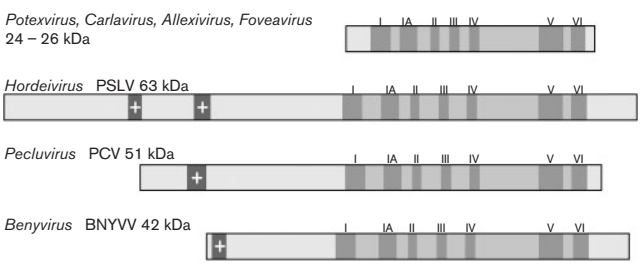

Fig. 2. Molecular organization of TGBp1. The dark grey region indicates the position of the helicase sequence domain with the seven conserved motifs I–VI. Black boxes labelled '+' indicate positively charged stretches in the N-terminal protein regions. PSLV, *Poa semilatent virus*; PCV, *Peanut clump virus*; BNYVV, *Beet necrotic yellow vein virus.*

comprises the entire sequence, whereas TGBp1s of rod-shaped viruses are substantially larger – from 39 to 63 kDa – and contain additional long N-terminal domains (Figs 1 and 2) (Solovyev *et al.*, 1996; Wong *et al.*, 1998; Erhardt *et al.*, 1999b). Peculiar features of these extensions are (i) the presence of arginine/lysine-rich clusters, possibly involved in binding of nucleic acids, and (ii) a region of sequence similarity just upstream of the helicase domain (Figs 2 and 3) (Bleykasten *et al.*, 1996; Solovyev *et al.*, 1996).

TGBp2 and TGBp3 contain hydrophobic sequences predicted to be involved in interaction of protein with membranes (Morozov *et al.*, 1987, 1989). All TGBp2s contain two hydrophobic segments, with a conserved central region between them (Fig. 4a), which exhibit the highest degree of sequence conservation among the TGB proteins (Morozov *et al.*, 1987; Skryabin *et al.*, 1988; Solovyev *et al.*, 1996).

Unlike TGBp2, which shows almost uniform molecular organization in viruses of different genera, sequences of TGBp3 form two main groups. In filamentous viruses of the genera *Potexvirus*, *Carlavirus*, *Foveavirus* and *Allexivirus*, the 6–13 kDa TGBp3 contains one hydrophobic sequence at the N terminus followed by a conserved region with the characteristic signature CX_5GX_8C (Fig. 4a) (Morozov *et al.*, 1991a). Another type of TGBp3 characteristic of rod-shaped viruses of the genera *Hordeivirus*, *Pomovirus* and *Pecluvirus* consists of 18–24 kDa proteins with two transmembrane segments, a conserved sequence in the N-terminal region containing invariant cysteine and histidine residues and the central conserved region with a typical tetrapeptide QDLN (Fig. 4a) (Solovyev *et al.*, 1996; Koenig *et al.*, 1998). Note that the conserved sequences in the TGBp3 hydrophilic regions of these two groups are not similar to one another. A third type of TGBp3 molecular organization is found in the genus *Benyvirus*, with two transmembrane segments but no significant sequence similarity with TGBp3 of the other rod-shaped viruses (Fig. 4a). Hence, a polyphyletic origin of TGBp3 can be proposed, whereas TGBp2, similarly to

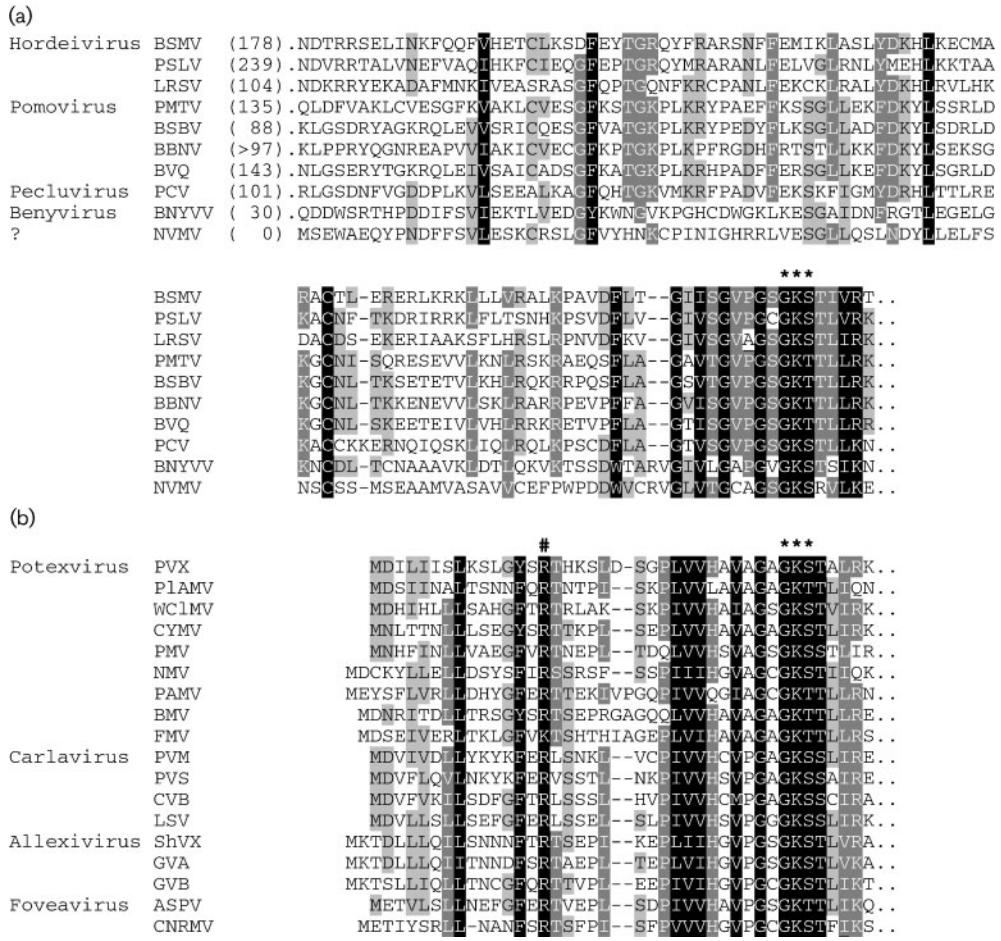

Fig. 3. Amino acid alignments of the sequences located upstream of conserved motif I of the TGBp1 helicase domain in hordei-like TGB (a) and potex-like TGB (b). Asterisks show the position of the conserved GKS tripeptide. The conserved arginine residue involved in RNA binding (see text) is indicated by '#'. Numbers in parentheses indicate distances from the N terminus. BSMV, *Barley stripe mosaic virus*; PSLV, *Poa semilatent virus*; LRSV, *Lychnis ringspot virus*; PMTV, *Potato mop-top virus*; BSBV, *Beet soil-borne virus*; BBNV, *Broad bean necrosis virus*; BVQ, *Beet virus Q*; PCV, *Peanut clump virus*; BNYVV, *Beet necrotic yellow vein virus*; NVMV, Nicotiana velutina mosaic virus; PVX, *Potato virus X*; PlAMV, Plantago asiatica mosaic virus; WClMV, *White clover mosaic virus*; CYMV, *Clover yellow mosaic virus*; PMV, *Papaya mosaic virus*; NMV, *Narcissus mosaic virus*; PAMV, *Potato aucuba mosaic virus*; BMV, *Bamboo mosaic virus*; FMV, *Foxtail mosaic virus*; PVM, *Potato virus M*; PVS, *Potato virus S*; CVB, *Chrysanthemum virus B*; LSV, *Lily symptomless virus*; ShVX, *Shallot virus X*; GVA, *Garlic virus A*; GVB, *Garlic virus B*; ASPV, *Apple stem pitting virus*; CNRMV, *Cherry necrotic rusty mottle virus*.

TGBp1 (Koonin & Dolja, 1993), most probably originates from a common ancestor (Solovyev *et al.*, 1996).

Based upon rules for topology of integral membrane proteins (Sipos & Von Heijne, 1993; Gafvelin *et al.*, 1997), hordeivirus TGBp2 and TGBp3 molecules are proposed to be integrated into the lipid bilayer in a U-like conformation with their N terminus exposed to the cytoplasm and endoplasmic reticulum (ER) lumen/extracellular space, respectively (Solovyev *et al.*, 1996). Another protein topology can be predicted for the potexvirus TGBp3s (Fig. 4b).

Thus, primary structure comparisons of the three TGB proteins allow us to distinguish two classes of TGBs: 'hordei-like' (class 1) and 'potex-like' (class 2) (Figs 1, 2 and 4) (Solovyev *et al.*, 1996; Erhardt *et al.*, 1999b). The biological relevance of this classification is confirmed by the different role played by viral coat protein (CP) in cell-to-cell movement of the viruses with different types of TGB (Callaway *et al.*, 2001). The CP is dispensable for cell-to-cell movement mediated by hordei-like TGBs (Petty & Jackson, 1990; Schmitt *et al.*, 1992; Herzog *et al.*, 1998; McGeachy & Barker, 2000). In contrast, the CP of potexviruses and, presumably, of other viruses with potex-like TGBs, is necessary for intercellular transport of the viral genome (Chapman *et al.*, 1992; Forster *et al.*, 1992; Sit & Abouhaidar, 1993) (see below).

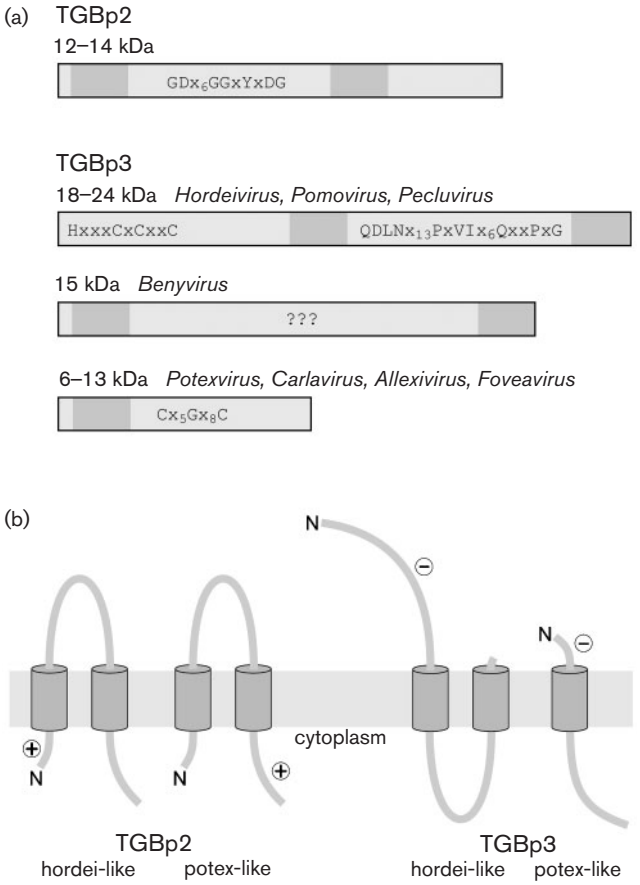

Fig. 4. Molecular organization of TGBp2 and TGBp3. (a) Conserved regions of the proteins. Molecular masses of proteins in different genera are shown. Dark grey boxes indicate hydrophobic transmembrane sequence segments. Characteristic signature sequences are shown. '???' indicates lack of information on sequence conservation in TGBp3 of benyviruses. (b) Predicted topology of TGBp2 and TGBp3 molecules in the cell membrane. Transmembrane helixes are depicted as cylinders; '+' and '−' show the net charge of hydrophilic regions of the proteins. The short N-terminal hydrophilic segment of hordei-like TGBp2 in rod-shaped viruses has a positive net charge of 2 to 3, the corresponding region of potex-like TGBp2 in filamentous viruses is neutral or has a positive net charge of 1. Conversely, the C-terminal hydrophilic segment in filamentous viruses possesses a net charge of 2 to 5.

Expression of TGB proteins in virus-infected plants

TGB proteins are expressed simultaneously at the early stages of infection (Niesbach-Klösgen *et al.*, 1990; Donald *at al.*, 1993), as are MPs of other viruses, such as TMV (Lehto *et al.*, 1990). Similarly to other Sindbis-like viruses, expression of 5′-distal genes in TGB-containing viruses occurs via subgenomic RNAs (sgRNAs) that are 3′ co-terminal with genomic RNAs (Buck, 1996; Agranovsky & Morozov, 1999). For TGBp1 and TGBp2, sgRNAs of the appropriate sizes have been found in infected plants, whereas TGBp3-specific sgRNA is not commonly detected (Guilford & Forster, 1986; Dolja *et al.*, 1987; Gilmer *et al.*, 1992; Zhou & Jackson, 1996). Detailed studies of TGB expression demonstrated that two sgRNAs are sufficient for translation of the three TGB proteins: the longer sgRNA serves as the template for translation of TGBp1, while the shorter sgRNA is the messenger for both TGBp2 and TGBp3 (Morozov *et al.*, 1991b; Zhou & Jackson, 1996; Verchot *et al.*, 1998; Agranovsky & Morozov, 1999). Expression of TGBp3 by leaky ribosome scanning through the TGBp2 gene was proposed (Skryabin *et al.*, 1988; Morozov *et al.*, 1989). This translation strategy maintains a low level of TGBp3 expression. For example, *in vitro* translation of a sgRNA transcript yields TGBp2 and TGBp3 in the ratio 10 : 1 (Zhou & Jackson, 1996). Furthermore, the expression level of TGBp3 in infected plants may be even lower, as suggested by the inability to detect TGBp3, while TGBp2 is detected easily (Niesbach-Klösgen *et al.*, 1990; Donald *at al.*, 1993; Gorshkova *et al.*, 2003).

Functions of TGBp1

Biochemical activities of TGBp1 *in vitro*

Cell-to-cell movement of plant viruses was postulated to involve specific non-virion transport RNPs (Atabekov & Dorokhov, 1984; Citovsky & Zambryski, 1993), and further experiments have demonstrated that all tested MPs of the '30K superfamily' are nucleic acid-binding proteins (Carrington *et al.*, 1996; Ghoshroy *et al.*, 1997; Tzfira *et al.*, 2000). The TGBp1 proteins, similarly to 30K superfamily MPs, can bind ssRNA non-specifically in a co-operative manner and have affinity for ssDNA as well (Rouleau *et al.*, 1994; Bleykasten *et al.*, 1996; Kalinina *et al.*, 2001; Donald *et al.*, 1997). For the potex-like TGBp1s, stability of *in vitro* co-operative binding to RNA is lower than for the 30K superfamily MPs (Rouleau *et al.*, 1994; Kalinina *et al.*, 1996, 1998; Lough *et al.*, 1998; Wung *et al.*, 1999). The RNA-binding site of potexviral TGBp1 has been mapped to the N-terminal protein region containing positively charged residues essential for interaction with RNA. One of these residues is an arginine, conserved in all potex-like TGBp1, 17–19 aa upstream of the GKS/T tripeptide (Fig. 3) (Morozov *et al.*, 1999; Wung *et al.*, 1999).

Multiple RNA-binding sites are found in hordeiviral TGBp1 (Donald *et al.*, 1995, 1997). The isolated C-terminal helicase domain of a hordeiviral TGBp1 shows co-operative RNA binding similar to that of potex-like TGBp1, while the N-terminal extension domain demonstrates strong non-co-operative RNA binding, so that the whole protein exhibits both types of binding (Kalinina *et al.*, 2001). Similarly, other hordei-like TGBp1s are capable of strong, salt-resistant RNA binding (Bleykasten *et al.*, 1996; Donald *et al.*, 1997; Cowan *et al.*, 2002). In hordeiviral TGBp1s, the two short arginine/lysine-rich regions are essential for N-terminal extension domain-specific RNA binding (Solovyev *et al.*, 1996; Kalinina *et al.*, 2001). Likewise, RNA

binding of a benyvirus TGBp1 is specified by an arginine/lysine-rich region positioned 6–18 residues from the N terminus (Fig. 2) (Bleykasten *et al.*, 1996).

Both potex- and hordei-like TGBp1s have RNA helicase activity *in vitro* (Kalinina *et al.*, 2002). Importantly, the hordeiviral TGBp1 helicase unwinds the duplex in both the 5′→3′ and the 3′→5′ directions, with respect to the chain used for entry, and is unable to unwind DNA duplexes (Kalinina *et al.*, 2002). In contrast, superfamily I (SF-I) DNA helicases and RNA virus SF-II helicases operate in the 3′→5′ direction only (Gorbalenya & Koonin, 1993; Kadare & Haenni, 1997). Generally, the duplex unwinding activity of helicases depends on the hydrolysis of NTPs, preferentially ATP (Gorbalenya *et al.*, 1989; Gorbalenya & Koonin, 1993; Kadare & Haenni, 1997; Soultanas & Wigley, 2001; Caruthers & McKay, 2002). Accordingly, the RNA helicase activity of TGBp1 requires ATP and Mg^{2+} (Kalinina *et al.*, 2002), and NTP-binding and Mg^{2+}-dependent NTPase activities have been detected for TGBp1 *in vitro* (Rouleau *et al.*, 1994; Bleykasten *et al.*, 1996; Kalinina *et al.*, 1996; Donald *et al.*, 1997; Morozov *et al.*, 1999; Solovyev *et al.*, 1999; Liou *et al.*, 2000). Finally, many helicases can form homodimers or oligomers (Gorbalenya & Koonin, 1993) and both hordei- and potex-like TGBp1 are capable of self-interactions (Cowan *et al.*, 2002; our unpublished data).

Four structural domains have been identified in SF-I DNA helicases. The N-terminal domain 1A includes helicase motifs I–III, while the C-terminal domain 2A carries motifs IV–VI. The sequences of domains 1A and 2A are interrupted by the inserted domains 1B and 2B (Fig. 5) (Soultanas & Wigley, 2001; Caruthers & McKay, 2002). Alignment with the sequence of PcrA, a bacterial SF-I DNA helicase with known three-dimensional structure, demonstrates that TGBp1 shows (i) conservation of the helicase motifs in domains 1A and 2A and (ii) the absence of domains 1B and 2B, which are precisely 'deleted' from the TGBp1 sequences (Fig. 5) (Kalinina *et al.*, 2002). Importantly, deletion of the 2B domain introduced in a bacterial SF-I DNA helicase (Rep protein) had no effect on helicase activity *in vitro* and *in vivo* (Cheng *et al.*, 2002). Hence, TGBp1 represents a naturally 'simplified' version of a SF-I helicase with just two structural domains. Interestingly, a similar structure has been described for the cellular eIF-4A helicase, the prototype member of the 'DEAD' SF-II family of RNA helicases, which shares with TGBp1 two other features exceptional among RNA helicases, namely the ability to discriminate between RNA and DNA and to operate in both directions (Gorbalenya & Koonin, 1993; Kadare & Haenni, 1997; Caruthers & McKay, 2002; Du *et al.*, 2002; Kalinina *et al.*, 2002).

Activities of TGBp1 *in vivo*

RNA-binding activity of TGBp1 is thought to be responsible for formation of movement-competent genomic RNPs; such structures composed of viral RNA and TGBp1 were isolated from hordeivirus-infected plants (Brakke *et al.*, 1988). Although the nature of movement-related RNPs in potexviruses remains obscure, they also contain TGBp1, which has been suggested to either interact with non-virion complexes that also contain CP (Lough *et al.*, 1998, 2000) or to bind to and modify virions in a manner that allows them to transport to and through PD (Fig. 6) (Santa Cruz *et al.*, 1998; Atabekov *et al.*, 2000).

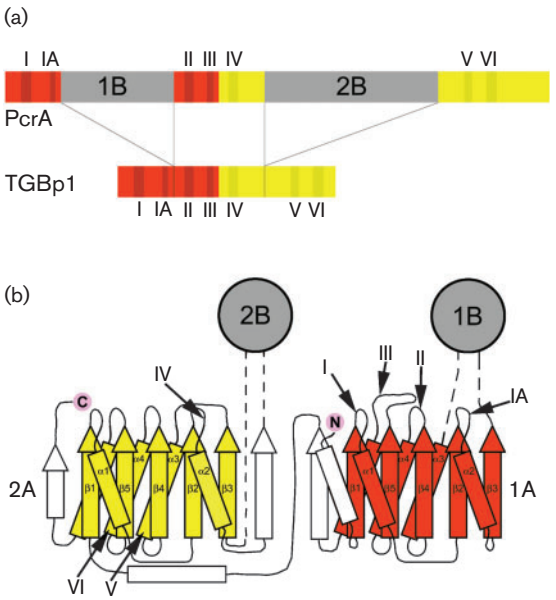

Fig. 5. Subdomain structure of TGBp1 helicases. (a) Comparison of TGBp1 helicase and PcrA, a bacterial DNA helicase. Conserved sequence motifs I–VI are indicated. Domains 1A and 2A are shown in red and yellow, respectively. Domains 1B and 2B, absent from TGBp1, are shown in grey. (b) Putative secondary structure of the helicase domain of TGBp1. In the modified model of the secondary structure of PcrA (Caruthers & McKay, 2002), sequence elements represented by parallel α–β structures and conserved in domains 1A and 2A of TGBp1 are shown in colour. Arrows point to the positions of conserved sequence motifs I–VI. Domains 1B and 2B, absent from TGBp1, are shown by grey circles.

The role of the other TGBp1 activities (NTP binding, NTPase and RNA helicase) in cell-to-cell movement remains unclear. According to recent views, the cell-to-cell movement of viral genomes is an energy-dependent process (Carrington *et al.*, 1996; Ghoshroy *et al.*, 1997). There are at least two steps of MP-mediated translocation of nucleic acids where such ATP/NTP-dependent events may be involved: (i) intracellular transport of MP and virus-specific RNP to PD or to a region in the vicinity of PD and (ii) trafficking of proteins and RNP through PD involving both protein/RNA unfolding and microchannel dilation (Fig. 6) (Ghoshroy *et al.*, 1997; Lazarowitz & Beachy, 1999; Lucas, 1999; Kragler *et al.*, 1998; Tzfira *et al.*, 2000; Haywood *et al.*, 2002; Heinlein, 2002b; Roberts & Oparka, 2003).

No ability to modify PD and move cell to cell has been

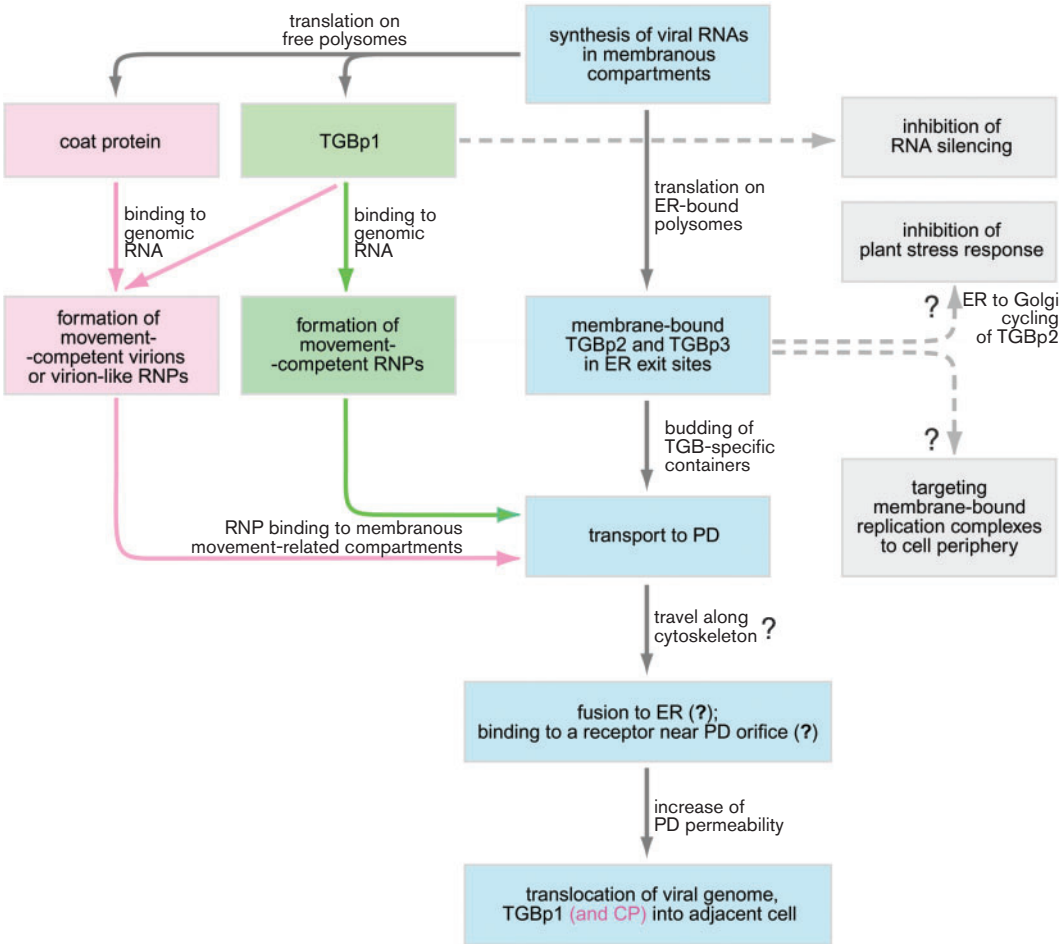

Fig. 6. General scheme of TGB-mediated cell-to-cell movement and formation of transport RNP complexes. Processes specific for potex-like and hordei-like TGBs are shown in pink and green, respectively. Transport steps common for both potex- and hordei-like TGBs are shown in blue. Processes that are not involved directly in virus cell-to-cell movement are shown in grey. Cell-to-cell movement initiates with the synthesis of virus genomic and subgenomic RNAs in the replication complexes located on membranes of the ER or other organelles (Plante *et al.*, 2000; Dunoyer *et al.*, 2002b; Schwartz *et al.*, 2002). TGBp1 is synthesized on free polysomes. Most probably, the hydrophobic TGBp2 and TGBp3 MPs enter the ER co-translationally, and the hydrophobic regions of TGBp2 and TGBp3 migrate into the ER membrane (Vitale & Denecke, 1999). TGBp2 and TGBp3 travel to their destinations in specific membrane containers (Jiang & Rogers, 1998; Mitsuhashi *et al.*, 2001; Stephens & Pepperkok, 2001; Nebenführ, 2002; Zamyatnin *et al.*, 2002). Trafficking to the cell periphery may exploit the cytoskeleton-based pathway (Ploubidou & Way, 2001; von Bargen *et al.*, 2001; Heinlein, 2002a). TGBp2/p3-specific membrane containers may bind movement-competent virions (or RNPs) containing TGBp1, either through protein–protein interactions or via direct interaction of TGBp2 and RNA (Solovyev *et al.*, 2000; Cowan *et al.*, 2002; Zamyatnin *et al.*, 2002). These complexes are delivered to the neck region of PD and fuse to cortical ER (Solovyev *et al.*, 2000; Zamyatnin *et al.*, 2002; Lee *et al.*, 2003). TGBp1 reaches the orifice of a PD microchannel and binds to receptor(s) involved in SEL increase (Lough *et al.*, 1998; Lucas, 1999; Lee *et al.*, 2003; Roberts & Oparka, 2003). TGBp1 and viral RNA are unfolded and then translocated through the PD microchannels until reaching the neighbouring cell (Kragler *et al.*, 1998; Lucas, 1999). For further details, see text.

reported for individually expressed hordei-like TGBp1, suggesting that this protein is incapable of intracellular trafficking to PD by itself and depends on TGBp2 and TGBp3 for this function (discussed below). Conversely, potexviral TGBp1 is capable of interacting with PD and increasing PD SEL (Angell *et al.*, 1996; Lough *et al.*, 1998, 2000; Malcuit *et al.*, 1999; Morozov *et al.*, 1999; Yang *et al.*, 2000). Mutations influencing the potexviral TGBp1 helicase motif I (disabling both helicase and NTPase activities of the protein) block the protein's ability to increase PD SEL, whereas mutations of motif VI (disabling the helicase but not NTPase activity) (Kalinina *et al.*, 2002) affect neither the increase in TGBp1-induced SEL nor TGBp1 transport to the cell periphery (Lough *et al.*, 1998; Morozov *et al.*, 1999).

Thus, ATP binding and/or hydrolysis rather than helicase activity per se are involved in PD dilation by potex-like TGBp1.

Potexviral TGBp1 is co-translocated with the CP and virus genomic RNA during virus movement through PD (Morozov *et al.*, 1997; Lough *et al.*, 1998, 2000, 2001). It is natural to propose that TGBp1 helicases couple RNA unwinding and translocation through PD microchannels. The discovery of a specialized RNA-translocating NTPase (P4 protein) that participates in RNA transfer and packaging into bacteriophage ϕ6 virions (Juuti *et al.*, 1998) further supports such a proposal.

TGBp1 of *Potato virus X* (PVX) has been reported to interact with one end of the virion and to induce energy-dependent conformational changes of virus particles *in vitro* (Atabekov *et al.*, 2000). Soultanas & Wigley (2001) have suggested that energy generated by ATP hydrolysis can be used by helicases not only for separation of base-paired regions but also for displacement of other proteins from nucleic acids. Thus, by analogy with some SF-II cell and viral helicases ('RNPases') involved in disassembly and remodelling of RNP complexes (Tseng *et al.*, 1998; Jankowsky *et al.*, 2001; Schwer, 2001), TGBp1 could potentiate cell-to-cell transport of virions or movement-related RNPs through PD by disrupting both RNA–protein interactions and intramolecular RNA base-pairing. In this case, in addition to trafficking of viral genomes through PD, hordei-like TGBp1 may mediate a displacement of cell proteins prior to genomic RNA transport to PD. During viral genome replication, nascent RNA molecules are most likely packaged into RNPs by host cytosolic proteins which rapidly coat newly synthesized cellular and virus-specific mRNAs and facilitate their efficient translation and further turnover (Mitchell & Tollervey, 2001; Pilipenko *et al.*, 2001; Fedoroff, 2002). Re-packaging of such RNPs by replacement of cell proteins with hordei-like TGBp1 may result in formation of movement-competent non-translatable RNPs (Karpova *et al.*, 1999).

TGBp1 as a factor of whole plant infection

CPs are dispensable for systemic infection of hordeiviruses and pomoviruses, which are thus believed to enter the phloem and traffick along the sieve tubes as a non-virion RNP containing genomic RNA and TGBp1 (Brakke *et al.*, 1988; Petty & Jackson, 1990; Donald *et al.*, 1997; McGeachy & Barker, 2000; Lawrence & Jackson, 2001b). In hordeiviral TGBp1, two positively charged motifs responsible for RNA-binding activity of the N-terminal extension domain (Fig. 2) have been found to be dispensable for virus transport from cell to cell but, nevertheless, necessary for long-distance virus movement. Therefore, the RNA-binding activities of the helicase and extension domains of hordei-like TGBp1 could be specialized in either cell-to-cell or long-distance transport, respectively (Kalinina *et al.*, 2001). However, the presence of the N-terminal extension in TGBp1 and its compatibility with the helicase domain are required to support both cell-to-cell and long-distance movement in hordeiviruses (Donald *et al.*, 1995, 1997; Solovyev *et al.*, 1999).

In contrast to hordeiviruses, a functional CP is required for potexvirus long-distance movement (Santa Cruz *et al.*, 1998). Potexviral TGBp1 is co-transported along the phloem sieve tube together with virions (or a non-virion CP-containing RNP). Because the sieve element contains no translational apparatus, this complex with TGBp1 must include all functional activities required for exiting from the sieve tube to the companion cells (Santa Cruz *et al.*, 1998; Lough *et al.*, 2001).

The ability of the virus to establish systemic infection in plants depends largely on the efficacy of plant defence response against infection versus the potential of the virus to escape or counter this defence (Carrington *et al.*, 2001; Dangl & Jones, 2001; Vance & Vaucheret, 2001). One of the defence mechanisms in plants is gene silencing, which is mediated by sequence-specific degradation of viral RNAs in the cytoplasm (Baulcombe, 2002; Voinnet, 2001; Waterhouse *et al.*, 2001). However, TGB-containing viruses, like many plant viruses, have evolved special mechanisms to suppress RNA silencing (Voinnet *et al.*, 2000; Dunoyer *et al.*, 2002a; Yelina *et al.*, 2002). In particular, potexviral TGBp1 has been shown to suppress production or activity of the mobile silencing signal (Voinnet *et al.*, 2000). Importantly, some of the sequences of TGBp1 involved in suppressing the silencing activity do not affect cell-to-cell movement (D. Baulcombe, The Sainsbury Laboratory, John Innes Centre, Norwich, UK, personal communication).

Another virus resistance mechanism is mediated by a large family of R gene-encoded proteins that recognize pathogen-encoded elicitors and trigger defence pathways, such as programmed cell death or hypersensitive response (Dangl & Jones, 2001; Holt *et al.*, 2003). PVX TGBp1 has been shown recently to be such an elicitor recognized by the *Nb* gene-mediated resistance system in potatos. The TGBp1 region required for activation of the *Nb* response is located in the N terminus upstream of the helicase motif I (Malcuit *et al.*, 1999).

Functions of TGBp2 and TGBp3

Subcellular distribution of TGBp2 and TGBp3 and virus movement

In agreement with sequence analysis (Fig. 4) and *in vitro* studies predicting that TGBp2 and TGBp3 are integral membrane proteins (Morozov *et al.*, 1987, 1990, 1991a), cell fractionation of plant tissues expressing these proteins demonstrates predominant association of both proteins with the P1 and P30 membranous fractions as well as with the cell wall (CW) fraction (Niesbach-Klösgen *et al.*, 1990; Donald *et al.*, 1993; Hefferon *et al.*, 1997; Cowan *et al.*, 2002; Gorshkova *et al.*, 2003).

Further studies of subcellular localization of TGBp2 and

TGBp3 employed their GFP fusions expressed in plant cells by a variety of techniques. Note that experiments on transient expression of MPs should be interpreted cautiously, since MPs expressed from vectors are likely produced in much larger quantities and in a non-regulated fashion compared to a virus infection. Also, methods of delivery of expression vectors such as high-pressure biolistic bombardment may perturb cell status (Crawford & Zambryski, 2001) and functional properties of proteins may be hindered by the fused fluorescent protein sequences (Thomas & Maule, 2000; Brandizzi *et al.*, 2002a). Nevertheless, transient expression of GFP fusions is widely used to study MPs in live cells and the results of such studies do not usually contradict data obtained by other methods (Lazarowitz, 1999; Lazarowitz & Beachy, 1999; Brandizzi *et al.*, 2002a; Heinlein, 2002a).

When transiently expressed in individual epidermal cells of *Nicotiana benthamiana*, GFP-tagged TGBp2s of *Poa semilatent virus* (PSLV) and *Potato mop-top virus* (PMTV) are localized to elements of the cell endomembrane system, mainly tubules of the cortical ER network (Solovyev *et al.*, 2000; Cowan *et al.*, 2002; Zamyatnin *et al.*, 2003). In cells with higher levels of PSLV TGBp2 expression, a part of the protein is also associated with motile vesicles (Solovyev *et al.*, 2000) that resemble plant Golgi stacks (Brandizzi *et al.*, 2002b). The Golgi-like mobile vesicles have been found to contain most of the transiently expressed TGBp2 of PVX (our unpublished data). Subcellular localization of TGBp2 to the ER and Golgi is determined by the hydrophobic protein segments and, in particular, the length of the C-terminal hydrophobic segment (Solovyev *et al.*, 2000; Zamyatnin *et al.*, 2002; our unpublished data), which seems to act as a retrieval signal in Golgi-to-ER recycling by the host receptor Rer1 (Sato *et al.*, 1999, 2001).

Transiently expressed individual GFP–TGBp3 is found in membrane bodies of different sizes located at the cell periphery in close association with the CW (Solovyev *et al.*, 2000; Cowan *et al.*, 2002). TGBp3 expression has little effect on the basic structure of the ER in plant cells. However, TGBp3 synthesis results in the formation of new TGBp3-containing ER structures (peripheral bodies) connected with the cortical ER network (Zamyatnin *et al.*, 2002; Gorshkova *et al.*, 2003). The size of these bodies correlates with the amount of TGBp3 protein produced in a given cell (Zamyatnin *et al.*, 2002). Thus, TGBp3 is able to induce formation (or proliferation) of a specific subdomain of the cortical ER.

A clue to the subcellular location of the TGBp3-containing bodies comes from fluorescent microscopy of leaves infected with a PMTV GFP–TGBp3-expressing virus vector (Cowan *et al.*, 2002) and transgenic plants expressing PSLV GFP–TGBp3 (Gorshkova *et al.*, 2003). When the protein is expressed in adjacent cells, the peripheral bodies formed in the two neighbouring cells are opposite to each other. The structural link that governs the formation of such twin bodies could be provided by PD (Cowan *et al.*, 2002) and specific staining of PD-associated callose confirms the localization of TGBp3-containing bodies alongside of PD (Gorshkova *et al.*, 2003).

Targeting of PSLV TGBp3 depends on a specific signal consisting of two parts, of which a central hydrophilic region conserved in all hordei-like TGBp3 (Fig. 4a) seems to be an oligomerization sequence (Cowan *et al.*, 2002; Gorshkova *et al.*, 2003; our unpublished data). Another part of the specific PD targeting signal of TGBp3 is located in the C-terminal transmembrane segment, which resembles a hydrophobic membrane-embedded segment that participates in forming a protein trafficking signal of mastrevirus MP (Kotlizky *et al.*, 2000). Similarly, localization of PVX TGBp3 to peripheral bodies depends on the only protein transmembrane segment (our unpublished data). Mutations in the hordeivirus TGBp3 signal result in localization of the protein in a 'granular network' of tiny bodies visible as a reticulate pattern as if they are formed on the surface of cortical ER tubules (Solovyev *et al.*, 2000; our unpublished data). Thus, it appears that mutations in either part of the bipartite signal permit protein segregation to ER-exit sites but cannot mediate further protein trafficking to the final destination at PD-associated compartments (Fig. 6).

Co-targeting of TGBp2 and TGBp3

In the presence of TGBp3, TGBp2 is re-targeted to peripheral bodies that resemble the structures observed in cells expressing TGBp3 (Solovyev *et al.*, 2000). Perfect co-localization of co-expressed PSLV TGBp2 and TGBp3 in peripheral bodies has been demonstrated, confirming that the TGBp3 protein directs subcellular targeting of TGBp2 from the ER network to sites of TGBp3 location (Zamyatnin *et al.*, 2002). PVX TGBp3 also targets PVX TGBp2 to peripheral bodies, showing that TGBp3-directed trafficking of TGBp2 occurs in both hordei- and potex-like TGBs (Solovyev *et al.*, 2000).

Protein–protein interactions that result in the formation of TGBp2–TGBp3 complexes could be the mechanism by which the two proteins are co-targeted to peripheral bodies. However, co-expression of TGBp2 and TGBp3 mutants failed to identify regions potentially responsible for the interaction between TGBp2 and TGBp3 molecules (Solovyev *et al.*, 2000). Further evidence for a sequence-independent co-targeting mechanism was obtained in experiments on co-expression of heterologous TGB proteins. Indeed, in spite of the absence of sequence similarity of TGBp3 proteins in hordei- and potex-like TGBs, PVX TGBp3 can target PSLV TGBp2 to peripheral bodies and PSLV TGBp3 can, likewise, target PVX TGBp2 (Solovyev *et al.*, 2000). Moreover, PSLV TGBp3 can also target totally unrelated membrane-bound MPs, such as the C4 protein of *Faba bean necrotic yellows virus* (genus *Nanovirus*) and the 6K protein of *Beet yellows virus* (genus *Closterovirus*), to peripheral bodies (Zamyatnin *et al.*, 2002). This suggests that a sequence-specific interaction of TGBp2 and TGBp3

molecules is unlikely to be involved in TGBp3-directed targeting of TGBp2 (Solovyev *et al.*, 2000; Zamyatnin *et al.*, 2002). Nevertheless, PMTV TGBp3 interacts physically with the homologous TGBp2 in a yeast two-hybrid system (Cowan *et al.*, 2002). Presumably, this interaction may depend on the residue composition of hydrophobic segments, enabling side chain interaction between membrane-embedded helices of proteins (Scholze *et al.*, 2002; Sjöberg & Garoff, 2003).

Note that TGBp3 apparently does not traffick any integral membrane protein, since GFP derivatives statically retained in ER membranes by synthetic hydrophobic anchors are not targeted by TGBp3 (Zamyatnin *et al.*, 2002). Thus, it appears that some functional feature(s), rather then a specific sequence, is responsible for efficient trafficking of membrane proteins by TGBp3. Such features could include specific localization and dynamics of the membrane proteins in the cell endomembrane system, including their ability to cycle between the ER and the Golgi (our unpublished data).

As noted above, an intermediate step of translocation of TGBp3 to PD involves its segregation in hypothetical 'TGBp3 islands' in ER membranes (ER-exit sites) (Fig. 6). These protein islands, which also include TGBp2, can be translocated using the targeting signal of TGBp3 to a specific receptor near PD in specific membrane containers (vesicles or tubules) (Stephens & Pepperkok, 2001; Nebenführ, 2002) delivered to the neck region of PD and fused there to cortical ER tubules (Fig. 6) (Solovyev *et al.*, 2000; Cowan *et al.*, 2002; Zamyatnin *et al.*, 2002; Gorshkova *et al.*, 2003).

Targeting of TGBp1 by TGBp2/TGBp3

Unlike potexviral TGBp1, which is capable of moving intracellularly to a peripheral layer of cytoplasm and PD (Lough *et al.*, 1998; Malcuit *et al.*, 1999; Morozov *et al.*, 1999; Yang *et al.*, 2000), hordei-like TGBp1 expressed individually is not targeted to specific sites at the cell periphery. However, when TGBp1 is expressed in the presence of other virus products, it localizes to the punctate structures at the CW (Erhardt *et al.*, 1999b, 2000; Lawrence & Jackson, 2001a). At higher magnification, these structures are visible as pairs of disconnected bodies on opposite sides of the CW, closely resembling the structures formed by GFP–TGBp3 in close vicinity to PD (see above). Accordingly, GFP–TGBp1 punctate bodies co-localized with callose (Erhardt *et al.*, 2000), confirming the immuno-gold detection of TGBp1 in PD of infected leaves (Erhardt *et al.*, 1999b). Experiments with chimeric virus genomes suggested TGBp2 and TGBp3 as the most probable components responsible for this localization. Indeed, a combination of TGBp1 with homologous TGBp2/TGBp3 was required for TGBp1 function, particularly trafficking to PD (Lauber *et al.*, 1998; Lough *et al.*, 1998, 2000; Erhardt *et al.*, 1999a, 2000; Solovyev *et al.*, 1999; Lawrence & Jackson, 2001a; Zamyatnin *et al.*, 2003). Hence, the role of TGBp2/TGBp3 may be primarily a matter of intracellular delivery of TGBp1-formed transport-competent RNPs to PD (Fig. 6).

There are indications that TGBp1 is actively, rather than passively, transported by TGBp2/TGBp3 to PD and that this process requires enzymatic activities of the protein. Mutations in the conserved sequence motifs in the NTPase/helicase domains of hordei-like TGBp1 not only blocked cell-to-cell movement of the virus but also abolished protein targeting to PD in the presence of TGBp2 and TGBp3 (Erhardt *et al.*, 2000; Lawrence & Jackson, 2001a; Zamyatnin *et al.*, 2003).

TGBp2-induced increase in PD permeability and other putative movement-related activities of TGBp2/p3 proteins

Some of the point and insertion mutants of TGBp2 are dominant–negative, i.e. they inhibit cell-to-cell movement and diminish virus accumulation (Beck *et al.*, 1994; Seppanen *et al.*, 1997; Lauber *et al.*, 2001). The recently discovered ability of potex- and hordei-like TGBp2 to facilitate movement of GFP between adjacent epidermal cells (Tamai & Meshi, 2001; our unpublished data) suggests that co-expression of a non-functional TGBp2 mutant during infection can interfere not only with viral RNP trafficking to PD but also with some additional movement-related function(s).

The molecular nature of the TGBp2-directed increase in PD permeability is enigmatic. However, a relationship between this phenomenon and modifications of the tissue stress-response system can be proposed. In particle bombardment studies, the ability of GFP, which is a 27 kDa protein, to spread from an initially transfected epidermal cell of source *N. benthamiana* leaves to neighbouring cells depends on experimental conditions. When the leaves of intact plants are bombarded, GFP spreads over multiple cell boundaries to give a focus of more than 30 fluorescent cells. However, GFP is confined mostly to single cells after bombardment of detached leaves in a vacuum chamber (Oparka *et al.*, 1999; Crawford & Zambryski, 2000, 2001; Itaya *et al.*, 2000; Krishnamurthy *et al.*, 2002). Under the latter conditions, TGBp2 potentiates the spread of GFP to adjacent epidermal cells (Tamai & Meshi, 2001). Various stress factors, including leaf detachment, are known to reduce PD SEL due to rapid callose deposition (Sivaguru *et al.*, 2000; Crawford & Zambryski, 2001; Radford & White, 2001; Roberts & Oparka, 2003). Hence, it is possible that TGBp2 expression is not involved directly in increasing PD permeability but rather significantly decreases callose deposition in the CW and can thus inhibit or reverse the stress-induced decrease in PD SEL (Fig. 6). In line with this hypothesis, potexvirus TGBp2 has been shown recently to interact with TIP, a host protein regulator of β-1,3-glucanase, which is a key enzyme of callose turnover (Fridborg *et al.*, 2003). Thus, keeping the PD neck region open by callose degradation (or prevention of callose

accumulation) is a possible function of TGBp2 at the early stage of infection (Fridborg *et al.*, 2003). In this context, it is interesting that co-expression of TGBp3 and TGBp2 completely blocks the TGBp2-induced 'increase' of PD SEL (our unpublished data). Probably, trapping of TGBp2 in the peripheral membrane bodies formed by TGBp3 in the vicinity of PD (see above) can either prevent interaction between TGBp2 and TIP or directly block intracellular trafficking of TIP.

Apart from trafficking of TGBp1 and genomic RNA and PD SEL control, small TGB proteins could be involved directly in regulating a hypersensitive response (Bleykasten-Grosshans *et al.*, 1997; Solovyev *et al.*, 1999; Lauber *et al.*, 2001; Kobayashi *et al.*, 2001). We believe that this activity of TGBp2/p3 could also be related to the regulation of callose turnover in view of the fact that (i) limitation of PVX spread in a hypersensitive response is accompanied by heavy callose deposits in the vicinity of PD (Allison & Shalla, 1974) and (ii) callose deposition may regulate virus movement in hypersensitive hosts by affecting the PD SEL (Iglesias & Meins, 2000; Bucher *et al.*, 2001; Crawford & Zambryski, 2001; Radford & White, 2001).

CONCLUSION

There is increasing evidence that viruses exploit endogenous intra- and intercellular trafficking pathways for spread of proteins and nucleic acids within plants. A growing number of plant cell proteins ['non-cell-autonomously acting plant proteins' (NCAPs)] has been demonstrated to have properties of plant virus MPs, such as the ability to increase PD SEL and traffic between cells. Moreover, some of these proteins are able to transport RNA through PD (Lucas, 1999; Crawford & Zambryski, 2000; Tzfira *et al.*, 2000; Blackman & Overall, 2001; Lucas *et al.*, 2001; Haywood *et al.*, 2002; Heinlein, 2002b; Lee *et al.*, 2003; Roberts & Oparka, 2003).

Importantly, TMV MP, similar to NCAP CmPP16, is not capable of PD modification and trafficking between cells in the absence of an assisting cell protein, NCAPP1 (Lee *et al.*, 2003). The dependence of TMV MP on NCAPP1 emphasizes the idea that viral MPs act in concert with a number of as yet undiscovered cell proteins required to accomplish intra- and intercellular steps in cell-to-cell movement. Thus, analysis of dissimilar virus transport systems that comprise several MPs may suggest possible roles of viral proteins that mimic components of host intracellular trafficking machinery.

TGBp1s share some features with NCAPs involved in plant development. First, ectopic expression of potexviral TGBp1 appears to cause defects in the cell-to-cell communications that control lateral organ development (Foster *et al.*, 2002). Second, TGBp1 competes directly for intercellular

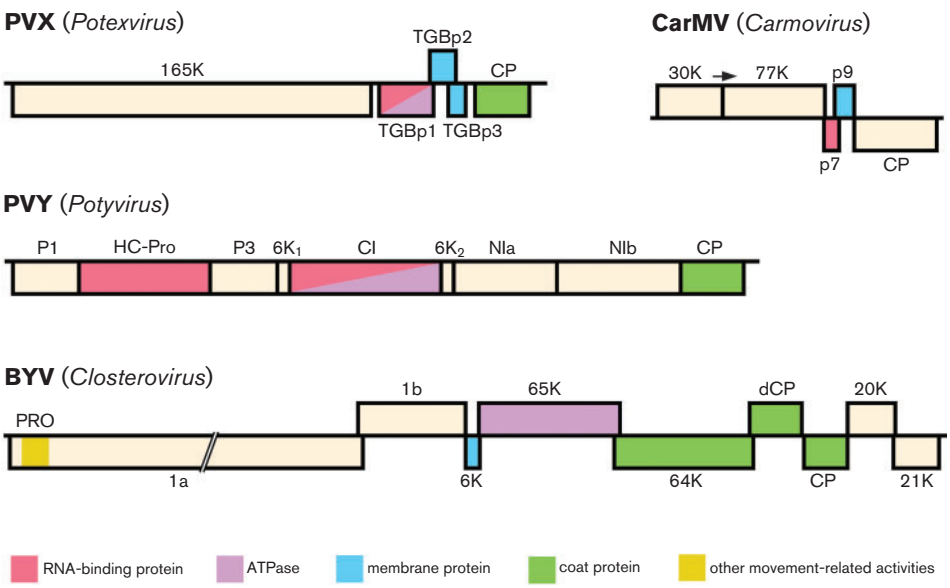

Fig. 7. Comparison of multicomponent cell-to-cell transport systems encoded by plant viruses. Genes are shown as boxes with names of encoded proteins. Genes of proteins involved in cell-to-cell movement are shown in colour. PRO, proteinase domain; PVX, *Potato virus X* (X05198); CarMV, *Carnation mottle virus* (X02986); PVY, *Potato virus Y* (M95491); BYV, *Beet yellows virus* (X73476). Potyviral proteins implicated in virus spread in addition to CI protein (see text) are the genome-linked proteins (VPg), HCpro and CP (Callaway *et al.*, 2001; Rajamaki & Valkonen, 1999; Revers *et al.*, 1999; Rojas *et al.*, 1997; Saenz *et al.*, 2002). BYV proteins involved in cell-to-cell movement in addition to Hsp70h (see text) include CP and its distant homologue (dCP), the minor CP 64K, the papain-like leader proteinase responsible for processing replicative protein precursors and the 6K small hydrophobic protein resembling potex-like TGBp3 (Alzhanova *et al.*, 2000; Peng *et al.*, 2001, 2002, 2003; Napuli *et al.*, 2003; Dolja, 2003).

trafficking pathways with NCAP Knotted-1 (Lough *et al.*, 2000). Third, the ability of NCAP CmPP16 to increase PD SEL and traffic through PD depends on an intracellular trafficking step directed by a membrane protein, NCAPP1, which localizes to cortical ER compartments in the vicinity of PD (Lee *et al.*, 2003), a pathway that parallels TGBp1 transport to PD-associated ER structures directed by TGBp2/TGBp3 (Fig. 6) (Zamyatnin *et al.*, 2002, 2003; Gorshkova *et al.*, 2003).

Carmo- and necroviruses (family *Tombusviridae*) have a transport system of two small MPs, an RNA-binding protein and a membrane protein (Fig. 7) (Hacker *et al.*, 1992; Marcos *et al.*, 1999; Vilar *et al.*, 2002). Therefore, in members of the family *Tombusviridae*, all energy-utilizing steps of movement likely depend on host proteins, a situation that is in contrast to TGB and other multi-component transport systems. Particularly, in members of the family *Potyviridae*, cell-to-cell movement requires the CI protein (Fig. 7), which is an SF-II helicase able to interact with PD and form conical deposits guiding potyviral filamentous virions to and through PD (Rodriguez-Cerezo *et al.*, 1997; Carrington *et al.*, 1998; Roberts *et al.*, 1998). However, it is not known whether the functions of the helicases in potexviruses and potyviruses (SF-I helicase TGBp1 and SF-II helicase CI) are similar. Viruses of the family *Closteroviridae* have no movement-related helicase but encode another ATPase, Hsp70h (Fig. 7), which is related to a large group of cell chaperones (Hsp70s) involved in energy-coupled processes of protein folding, degradation and transport (Agranovsky *et al.*, 1991, 1997; Ellis & Hartl, 1999; Pilon & Schekman, 1999). Hsp70h is required for cell-to-cell and long-distance movement as well as for infectivity of virus particles and assembly of movement-competent virions (Agranovsky *et al.*, 1998; Medina *et al.*, 1999; Peremyslov *et al.*, 1999; Napuli *et al.*, 2000; Alzhanova *et al.*, 2001; Prokhnevsky *et al.*, 2002; Dolja, 2003). Similarly to TGBp1 and TGBp2/TGBp3 proteins (which mimic functions of NCAPs and NCAPP1, respectively), closteroviral Hsp70h may be the virus counterpart of a host component of a cell-to-cell movement machine.

Recently, Aoki *et al.* (2002) identified a new subfamily of cell Hsp70 proteins that exhibit properties of NCAPs, including the ability to interact with PD. Another type of cell chaperone has been shown to interact with the MP of *Tomato spotted wilt virus* (von Bargen *et al.*, 2001). Additionally, translocation of NCAP Knotted-1 through PD requires partial protein unfolding (Kragler *et al.*, 1998; Roberts & Oparka, 2003), suggesting the role of cell chaperones at this step of host- and virus-specific cell-to-cell movement (Fig. 6). In general, it can be speculated that plant viruses have acquired and adopted distinct components of the cell trafficking machinery. As a result, these adaptive evolutionarily events have allowed viruses to recruit the existing host pathways of intra- and intercellular transport.

ACKNOWLEDGEMENTS

We are grateful to Dr D. Baulcombe for providing unpublished data and to Dr N. S. Vassetzky for critical reading of the manuscript. Work of the authors was supported in part by Volkswagen-Stiftung, Federal Republic of Germany.

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