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, Pomoivirus 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.

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 helicas of alpha-like viruses and belongs to helicas of superfamily I (SF-1) (Fig. 2) (Gorbunova et al., 1989; Gorbunova & Koonin, 1993; Koonin & Dolja, 1993). Of seven typical motifs in this
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, Carlaviruses, Foveavirus and Allexivirus) and rod-shaped viruses (genera Hordeiviruses, Benyviruses, Pomoviruses and Pecluviruses). Furthermore, the molecular masses of TGBp1 in filamentous viruses range from 24 to 26 kDa and the NTPase/helicase domain 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, Carlaviruses, 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$_{5}$GX$_{4}$C (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 hydrophobic regions of these two groups are not similar to one another. A third type of TGBp3 molecular organization is found in the genus Benyivirus, 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
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).
For TGBp1 and TGBp2, sgRNAs of the terminal with genomic RNAs (Buck, 1996; Agranovsky & Morozov, 1999). 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 (Skrabayin 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 et 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

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 et 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 (Guillford & 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 (Skrabayin 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 et al., 1993; Gorshkova et al., 2003).

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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 (Kalina 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 (Kalina et al., 1998). In contrast, superfamly 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 Mg2+ (Kalina et al., 2002), and NTP-binding and Mg2+-dependent NTPase activities have been detected for TGBp1 in vitro (Rouleau et al., 1999; Bleykasten et al., 1996; Kalina 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) (Kalina 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 elf-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; Kalina 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).

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; Tzifra et al., 2000; Haywood et al., 2002; Heinlein, 2002b; Roberts & Oparka, 2003). No ability to modify PD and move cell to cell has been...
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 (‘RNases’) 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 traffic 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; McGeeachy & 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 potatoes. 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; Hefferson 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 transmembrane segment, which resembles a hydrophobic membrane-embedded segment that participates in forming a protein trafficking signal of mastrevirus MP (Kotlízky 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 colocalization 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

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 (Kotlízky 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).
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 traffic 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; Gorschikova 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; Tzifira 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 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 Cl 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 helicas in potexviruses and potyviruses (SF-I helicase TGBp1 and SF-II helicase Cl) 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 & Opara, 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 adaptively evolutionarily events have allowed viruses to recruit the existing host pathways of intra- and intercellular transport.

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