CURRENT REVIEW

Varied Movement Strategies Employed by Triple Gene Block–Encoding Viruses

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Several RNA virus genera belonging to the *Virgaviridae* and *Flexiviridae* families encode proteins organized in a triple gene block (TGB) that facilitate cell-to-cell and long-distance movement. The TGB proteins have been traditionally classified as hordei-like or potex-like based on phylogenetic comparisons and differences in movement mechanisms of the *Hordeivirus* and *Potexvirus* spp. However, accumulating data from other model viruses suggests that a revised framework is needed to accommodate the profound differences in protein interactions occurring during infection and ancillary capsid protein requirements for movement. The goal of this article is to highlight common features of the TGB proteins and salient differences in movement properties exhibited by individual viruses encoding these proteins. We discuss common and divergent aspects of the TGB transport machinery, describe putative nucleoprotein movement complexes, highlight recent data on TGB protein interactions and topological properties, and review membrane associations occurring during subcellular targeting and cell-to-cell movement. We conclude that the existing models cannot be used to explain all TGB viruses, and we propose provisional *Potexvirus*, *Hordeivirus*, and *Pomovirus* models. We also suggest areas that might profit from future research on viruses harboring this intriguing arrangement of movement proteins.

Over the past 20 years, several paradigms have arisen that describe the mechanisms used by plant viruses to spread from infected cells into uninfected tissue via plasmodesmata (Pd). It is now evident that all plant viruses encode movement proteins that function to facilitate transit of infectious entities from the sites of replication through the endomembrane system and then dilate or “gate” Pd to enable movement of proteins, viral genomes, or virions into neighboring cells. *Tobacco mosaic virus* (TMV) encodes a single movement protein (designated 30K) and studies of this protein led to the first model explaining virus movement. The TMV 30K protein binds RNA cooperatively to form a ribonucleoprotein (RNP) complex that is transported across gated Pd into neighboring cells, whereupon the viral genome continues further rounds of translation and

replication. Subsequently, there have been numerous reports of similar functions for other virus-encoded movement proteins which have led to a proposed “30K superfamily” (Melcher 2000), as well as a universal model to explain intercellular transport mechanisms employed by viruses encoding 30K-related proteins. In addition, virus–host interactions involved in cell-to-cell movement of 30K protein have been reviewed (Epel 2009a; Heinlein and Epel 2004; Taliansky et al. 2008). A second paradigm that has not been well studied relates to some small, spherical RNA viruses, which encode two small, overlapping movement proteins that function in concert with the coat protein (CP) to facilitate cell-to-cell movement (Cheng et al. 1998; Kasteel et al. 1997; Sanchez-Navarro et al. 2010). A third distinct mechanism is employed by a group of icosahedral, pleiomorphic, or bacilliform plant viruses encoding one or two movement proteins that assemble into a hollow tubule that extends across the Pd. Electron micrographs have shown that, for several of these viruses, such as *Cowpea mosaic virus*, *Grapevine fan leaf virus*, *Cauliflower mosaic virus*, and *Tomato spotted wilt virus*, the tubules serve as conduits for transfer of virions or nucleoprotein cores into neighboring cells (Carvalho et al. 2004; Cheng et al. 1998; Kasteel et al. 1993, 1997; Laporte et al. 2003). Finally, studies of the family *Closteroviridae*, and *Beet yellows virus* in particular, are elucidating another model in plant virus transport. These viruses are unique due to their large RNA genomes and exceptionally long filamentous virions and require a five-component machinery for cell-to-cell movement (Dolja et al. 2006).

The topic of this review concerns mechanisms utilized by a number of diverse helical plant RNA viruses that encode triple gene block (TGB) movement proteins (Jackson et al. 2009; Morozov and Solovyev 2003; Morozov et al. 1989; Verchot-Lubicz et al. 2007). The TGB is a specialized, evolutionarily conserved genetic module that exploits the coordinated actions of three nonstructural viral proteins to deliver viral genomes to and through the Pd into neighboring cells (Fig. 1). Phylogenetic comparisons have revealed two major classes of TGB modules, potex-like and hordei-like (Morozov and Solovyev 2003). Although a single diagrammatic model was put forward to explain the movement of TGB-containing viruses, only limited comparative research has been conducted to determine whether intracellular trafficking pathways, from the site of virus replication to and across the Pd, are conserved among members of the hordei-like and potex-like TGB groups or even across genera. Many

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functions are conserved among the TGB-containing viruses studied to date; profound differences also exist, including varying requirements for the viral CP for movement and differences in the structure and functions of TGB proteins. A few studies

also indicate that the cellular protein requirements for function of the TGB proteins may differ among virus genera (Table 1). The goal of this article is to review the current knowledge about the mechanisms of TGB-assisted movement of virus

Triple gene block containing members of Flexiviridae

Proposed Alphaflexiviridae

Proposed Betaflexiviridae

Triple gene block containing members of Virgaviridae

Hordeivirus

Peculivirus

Pomovirus

Unassigned Virus

Benyvirus

Subgenomic RNA expression is similar for most TGB viruses

genomes, and to update previous models advanced to explain movement mechanisms of different TGB viruses. We note that there are significant differences between *Pomovirus*, *Hordeivirus*, and *Potexvirus* spp. which lead us to propose separate models to explain the transport for members of these genera. Given the phylogenetic relationships of *Hordeivirus* and *Pomovirus* TGB proteins, there are unique properties that warrant considering distinct models to explain the cell-to-cell transport of these different viruses. We also discuss areas where comparable information is lacking among the various virus genera and propose directions for future research.

Overview of TGB-encoding viruses.

The TGB-containing viruses belong to nine genera within the families *Alphaflexiviridae*, *Betaflexiviridae*, and *Virgaviridae*, as well as the unassigned genus *Benyvirus* (Fig. 1; Table 1). Viruses classified in the *Alphaflexiviridae* and *Betaflexiviridae* have monopartite genomes and encode the potex-like group of TGB proteins whereas TGB-encoding viruses within the *Virgaviridae*, which include the genera *Hordeivirus*, *Pomovirus*, and *Pecluvirus*, have two to three genome segments and encode hordei-like TGB. Members of the unassigned *Benyvirus* genus contain four or five genome segments (Lennfors et al. 2005; Peltier et al. 2008; Schirmer et al. 2005). Some members of the *Flexiviridae* and *Virgaviridae* encode 30K-like movement proteins. This variation in utilization of 30K-like or TGB-based movement strategies within members of the same family reinforces the idea of evolutionary models based on genome recombination (Morozov et al. 1989) and demonstrate that different classes of movement proteins have the potential to adapt quickly to facilitate efficient virus spread. Moreover, both 30K (or other single movement protein [MP]-based) and TGB transport systems can support movement of artificial recombinants, suggesting at least a partial independence of replication and movement functions (Ajikuttira et al. 2005; Atabekov et al. 1999; Lauber et al. 1998; Solovyev et al. 1996, 1997; Tamai et al. 2003; Xiong et al. 2005). Nevertheless, shuffling of individual cistrons between TGB proteins of different origin, in cis versus in trans expression from different mRNAs, or alterations in the relative ratios of TGB protein expression usually result in blockage or dramatic reductions in movement functions (Bleykasten-Grosshans et al. 1997; Lim et al. 2008; Lin et al. 2007; Lauber et al. 1998; Morozov et al. 1999; Solovyev et al. 1999). Most TGB findings are derived from studies of eight model viruses listed in Table 1 (Morozov and Solovyev 2003). A better understanding of the divergent roles of TGB proteins from various origins can contribute to our understanding of TGB mechanisms and will illuminate more global features of the movement processes of the wider group of viruses.

Common and divergent features of TGB proteins.

In all virus genera, the TGB open reading frames partially overlap and encode three proteins designated TGB1, TGB2,

and TGB3 (Fig. 1). Although the arrangement of the TGB cistrons and their modes of expression are conserved, the genome organization of individual viruses harboring TGB proteins varies considerably (Gilmer et al. 1992; Morozov and Solovyev 2003; Verchot-Lubicz et al. 2007; Jackson et al. 2009) (Fig. 1; Table 1). Most of the TGB proteins are expressed from two subgenomic (sg)RNAs that are co-terminal with the 3' end of the viral genome (Verchot et al. 1998; Zhou and Jackson 1996). The largest TGB1 protein is expressed from a high-abundance genomic (*Pomovirus* spp.) or sgRNA1, whereas TGB2 and TGB3 proteins are co-translated from a lower-abundance sgRNA (Fig. 1). The TGB3 protein is expressed via leaky ribosome scanning through the TGB2 start codon on the sgRNA. *Barley stripe mosaic virus* (BSMV) expression of the sgRNAs is temporally controlled and the two mRNAs accumulate to $\approx 10:1$ ratios of sgRNA1 to sgRNA2. The ratio for accumulation of TGB1, TGB2, and TGB3 proteins is estimated to be 100:10:1, respectively (Jackson et al. 2009; Johnson et al. 2003). Temporal control of TGB expression, sgRNA ratio, or protein accumulation have not been investigated in detail with other TGB-containing viruses. However, disruption of the ratios of TGB2:TGB3 proteins leading to overexpression of TGB3 relative to TGB2 has been shown to interfere with Pd targeting and cell-to-cell movement of *Beet necrotic yellow vein virus* (BNYVV), BSMV, and *Potato mop-top virus* (PMTV) (Bleykasten-Grosshans et al. 1997; Lim et al. 2008), suggesting similar requirements for temporal expression of the TGB.

TGB1 protein.

The hordei-like and potex-like TGB1 proteins differ substantially in their molecular mass and composition (Morozov and Solovyev 2003). The hordei-like TGB1 proteins range in size from 42 to 63 kDa and consist of three distinct structural and functional domains designated the N-terminal domain (NTD), the internal domain (ID), and a helicase-like domain (HELD) at the C terminus of the protein (Makarov et al. 2009). In contrast, the potex-like TGB1 proteins are substantially smaller (≈ 25 kDa) and lack the NTD and ID extensions; however, they contain an HELD region and an important region upstream consisting of 25 amino acids at the N terminus. This latter domain includes three conserved arginine residues that are necessary for *Bamboo mosaic virus* (BaMV) TGB1 protein ATPase activity, RNA binding, and cell-to-cell movement (Lin et al. 2004; Liou et al. 2000; Wung et al. 1999). Interestingly, the TGB1 C-terminal HELD regions of the *Hordeivirus*, *Benyvirus*, *Pomovirus*, and *Pecluvirus* genera are related to helicases found in the replicases of the family *Alphaflexiviridae*. Whatever their origin, mutations within conserved TGB1 helicase motifs I, Ia, and II reveal that the helicase domain functions in homologous interactions, cooperative RNA binding, RNA helicase functions, ATP and Mg^{2+} binding, and NTPase activities (Bleykasten et al. 1996; Donald et al. 1997; Han et al. 2007, 2009; Kalinina et al. 2001, 2002; Lawrence

Fig. 1. Genome organization of triple gene block (TGB)-containing viruses. Boxes represent genome-encoded open reading frames. Replicase gene domains are indicated in the white boxes: M, methyl transferase; A, AlkB; O, OTu-like peptidase; P, papain-like protease; H, RNA helicase; R, RNA-dependent RNA polymerase. Orange boxes represent the TGB. The largest gene on the left is TGB1. The TGB2 coding sequence overlaps TGB1 at the 3' end and TGB3 overlaps TGB2 at the 3' end. H indicates helicase domains. Pink boxes indicate RNA binding proteins and blue boxes specify the viral coat proteins (CPs). Certain replicase or CP proteins have a read-through domain, indicated by a line within the boxes. For *Beet necrotic yellow vein virus* (BNYVV), the replicase is released by a self cleavage mechanism. For *Potato mop-top virus* (PMTV), the CP read-through domain is required for vector transmission. The TGB-containing viruses belong to nine genera within the families *Flexiviridae* and *Virgaviridae*, as well as the unassigned genus *Benyvirus*. Members of the *Flexiviridae* family include *Potexvirus*, *Allexivirus*, *Mandarivirus* (in the proposed family *Alphaflexiviridae*), *Carlavirus*, and *Foveavirus* (in the proposed family *Betaflexiviridae*) species (Adams et al. 2004; Martelli et al. 2007). The bottom left box (outlined in black) shows the subgenomic (sg)RNA expression strategy employed by most TGB-containing viruses, except for the *Pomovirus* spp., in which TGB1 is expressed from gRNA-TGB. The top portion of the sgRNA box illustrates a fragment of the gRNAs that encode the TGB modules. TGB sgRNA1 and sgRNA2 are 3' coterminal; however, sgRNA1 is monocistronic and is responsible for expression of TGB1. sgRNA2 is bicistronic and is responsible for expression of TGB2 and TGB3.

and Jackson 2001a; Leshchiner et al. 2006; Li et al. 2001; Lim et al. 2008, 2009; Lin et al. 2004; Liou et al. 2000; Rouleau et al. 1994; Solovyev et al. 2000; Wung et al. 1999; Verchot-Lubicz et al. 2007).

Secondary structure predictions and circular dichroism spectra analyses of isolated NTD and ID domains of *Poa semilatifolia* virus (PSLV) indicate that the NTD is unfolded, whereas the ID has a pronounced secondary structure (Makarov et al. 2009). In vitro RNA-binding assays and deletion analysis have also revealed that RNA binding is inhibited by mutations in the N-terminal domain for BNYVV, BSMV, PSLV, and PMTV (Bleykasten et al. 1996; Donald et al. 1997; Kalinina et al. 2001). The BSMV and PSLV TGB1 proteins have multiple

RNA-binding regions that function at high ionic strength, and various TGB1 protein domains have different preferences for single-stranded (ss)RNA versus double-stranded (ds)RNA binding (Donald et al. 1997; Kalinina et al. 2001). Recent evidence has indicated that the NTD interacts with RNA noncooperatively (Makarov et al. 2009), and this binding presumably involves two clusters of positively charged amino acid residues (Donald et al. 1997; Jackson et al. 2009; Kalinina et al. 2001). TGB1 NTD, ID, and HELD domains are capable of self-interactions (Kalinina et al. 2001; Makarov et al. 2009), and the isolated ID is able to interact in solution with both the NTD and the C-terminal HELD domains (Makarov et al. 2009). Interactions between the three TGB1 RNA-binding domains

Table 1. Overview of triple gene block (TGB)–encoding viruses

Order	Monopartite					Multipartite			
	<i>Tymovirales</i>					Unassigned			
	<i>Alphaflexiviridae</i> ^a		<i>Betaflexiviridae</i> ^a			<i>Virgaviridae</i> ^a			Unassigned
Family									
Genus	<i>Potexvirus</i>	<i>Allexivirus</i>	<i>Mandarivirus</i>	<i>Carlavirus</i>	<i>Foveavirus</i>	<i>Hordeivirus</i> ^b	<i>Pomovirus</i>	<i>Pecluvirus</i>	<i>Benyvirus</i>
Primary species used in TGB studies ^c	PVX, WCIMV, BaMV					BSMV, PSLV	PMTV	PCV	BNYVV
Coat protein (CP)–required for:									
Cell-to-cell movement	Yes, filamentous ^d					No, rod-shaped			
Long-distance movement	Yes					No	No	Yes	Yes
TGB1	Viral RNA-binding protein; NTPase/helicase activity								
	Low molecular mass of ~25 kDa Increases Pd SEL ^e Can move independently RNA silencing suppressor					High molecular mass of ~42 to 63 kDa with N-terminal extension Does not increase Pd SEL Does not move independently (requires TGB2 or TGB3) No silencing suppressor activity detected but supported by: γ ^b p15 p14 & p31			
	Binds to the 5' end of RNA in particles and promotes structural rearrangement					Interacts with CP-RT ^f			
TGB2 ^g	Two predicted transmembrane (TM) domains								
	12- to 13-kDa membrane spanning protein					13- to 14-kDa membrane protein Endoplasmic reticulum (ER) and motile granules associated			
	Membrane association implicated in movement Induces proliferation of post-ER vesicles					Associated with:			
						Replicase	Components of endocytic pathways		ER-derived membrane structures near Pd
	No evidence of endocytic localization					Does not target Pd independently	Increases Pd SEL		
	Aids Pd gating in some hosts Interacts with TIP, a β -1,3-glucanase interactor that regulates callose accumulation						Unspecific ssRNA binding		
TGB3 ^g	7 to 8 kDa Single N-term TM domain					17 kDa	21 kDa	17 kDa	15 kDa
	Associates with ER and post-ER vesicles					Two TM domains			
	Alongside replicase in membrane-bound bodies					Contains:			
						C terminal and membrane spanning residues for PD targeting	Pd targeting signal: YQDLN and TM2 domain		
						Colocalizes with At4/1 at Pd (PSLV)	Increases Pd SEL		

^a It should be noted that some members of these families encode a single movement protein related to the '30K' superfamily (Melcher 2000; Martelli et al. 2007) instead of a TGB, and these viruses are not included in this review.

^b *Hordeivirus* TGB1, TGB2, and TGB3 proteins are formerly designated β b, β c, and β d, respectively.

^c *Potato virus X* (PVX), *White clover mosaic virus* (WCIMV), *Bamboo mosaic virus* (BaMV), *Barley stripe mosaic virus* (BSMV), *Poa semilatifolia virus* (PSLV), *Potato mop-top virus* (PMTV), *Peanut clump virus* (PCV), and *Beet necrotic yellow vein virus* (BNYVV).

^d *Tymovirales* order also contains icosahedral viruses.

^e Pd SEL = plasmodesmata size exclusion limit.

^f CP-RT = coat protein read-through domain.

^g TGB 2 and TGB3 are integral membrane proteins.

could provide the basis for a remodeling of TGB1-formed RNP complexes (discussed below) during different phases of virus cell-to-cell and long-distance transport (Makarov et al. 2009).

The multifunctional *Potexvirus* TGB1 proteins have some functions not found in hordei-like TGB1 proteins (Table 1). In addition to RNA binding and helicase activities, the *Potexvirus* TGB1 proteins increase Pd size exclusion limits (SEL). The TGB1 proteins of *White clover mosaic virus* (WCIMV) and *Potato virus X* (PVX) enabled the transfer of 10-kDa F-dex-trans between cells, which were restricted to single cells in the absence of TGB1 or in the presence of ATPase-deficient TGB1 (Angell et al. 1996; Howard et al. 2004; Lough et al. 1998). Biolistic bombardment studies also showed that green fluorescent protein (GFP)-TGB1 fusions spread to a greater percentage of cells than GFP in four different plant species (Howard et al. 2004), although TGB1 did not aid transport of GFP in trans (Tamai and Meshi 2001). The Arrhenius equation was employed to calculate the activation energy for transport by examining the temperature dependence of the transport process (Hille 1992). These data indicates that GFP as well as GFP-TGB1 movement across Pd is passive and involves a diffusion-driven process with a significantly lower activation energy (≤ 30 kJ/mol) compared with active (ATP driven) transport processes (≥ 50 kJ/mol) (Schoenkecht et al. 2008). Similar results have been reported for the TMV GFP-30K fusion protein (Liarzi and Epel 2005). However, the exact role of TGB1 ATPase activity in Pd transport remains unknown and, because different experimental approaches gave rise to contradictory results for the *Potexvirus* TGB1 protein cell-to-cell movement and its ability to increase SEL, additional studies are required to reconcile the existing data.

Additional distinctive functions of *Potexvirus* TGB1 proteins include silencing suppression and promoting translation of virion-derived RNAs (Atabekov et al. 2000; Bayne et al. 2005; Rodionova et al. 2003; Senshu et al. 2009). Silencing suppression activity has not been identified for *Hordeivirus* TGB1 proteins, despite their abilities to bind dsRNAs tenaciously (Jackson et al. 2009). Most of these latter viruses encode dedicated non-TGB silencing suppressors which are dispensable for cell-to-cell movement (Bragg et al. 2004; Donald and Jackson 1994; Yelina et al. 2002, 2005). Similarly, silencing suppression and movement functions appear to be uncoupled in the potex-like TGB1 multifunctional proteins (Bayne et al. 2005; Lim et al. 2010). Potex-like TGB1 proteins have been reported to enable disassembly of virions and to promote vRNA translation (Atabekov et al. 2000; Lukashina et al. 2009; Rodionova et al. 2003). It is reasonable to consider that this activity is essential for transport of TGB1-vRNA-CP complexes across Pd into the receiving cell where the first step in the virus replication cycle is translation. Further research is needed to determine whether the role of TGB1 in vRNA translation is linked to virus movement.

TGB2 and TGB3 proteins.

TGB2 and TGB3 proteins are integral membrane proteins (Fig. 2). TGB2 proteins range in mass between 12 and 14 kDa, share much sequence similarity, and have two predicted hydrophobic transmembrane segments with a central conserved hydrophilic loop (Morozov and Solovyev 2003). A topological model, supported by experimental data for *Potexvirus*, *Hordeivirus*, and *Pomovirus* spp., predicts that TGB2 proteins integrate into membranes in a U-shape with the central loop exposed to the endoplasmic reticulum (ER) lumen (Hsu et al. 2008; Martelli et al. 2007; Mitra et al. 2003; Morozov and Solovyev 2003; Zamyatnin et al. 2006). Although the short hydrophilic TGB2 protein sequences contain no signature motifs representative of known enzymatic or structural domains, functional

activities of TGB2 proteins have been reported for several viruses. In particular, the PMTV and BaMV TGB2 proteins have been reported to bind RNA in vitro in a sequence-nonspecific manner (Cowan et al. 2002; Hsu et al. 2009). The PVX TGB2 protein also has been reported to increase Pd SEL, as judged from biolistic experiments in which cell-to-cell diffusion of cytosolic GFP was enhanced considerably upon GFP coexpression with TGB2 (Tamai and Meshi 2001) (A. G. Solovyev and S. Yu Morozov *unpublished*). Similarly, mRFP-fused TGB2 of PMTV has been reported to increase Pd SEL and enable movement of a GFP-sporamin fusion protein into neighboring cells (Haupt et al. 2005).

TGB3 protein sequences are the most poorly conserved across virus genera, suggesting either radically divergent speciation or a polyphyletic origin (Martelli et al. 2007; Morozov and Solovyev 2003). The larger (18 to 24 kDa) *Virgaviridae* TGB3 proteins have two transmembrane domains that are conserved in BSMV, PSLV, and PMTV as well as N-terminal sequences containing invariant cysteine and histidine residues, and a central hydrophilic region containing the YQDLN motif conserved in *Hordeivirus* and *Pomovirus* spp. but absent in *Peculivirus* and *Benyvirus* spp. (Solovyev et al. 1996). Previously, computer algorithms predicted that the N and C termini were located in the ER lumen. However, recent experimental evidence indicates that the N and C termini of PMTV and PSLV TGB3s are in the cytoplasm and the loop regions protrude into the lumen (Tilsner et al. 2010) (E. A. Shemyakina, A. G. Solovyev, and S. Yu Morozov *unpublished data*). This is supported by recent predictions based on Hidden Markov Models (Kall et al. 2004; Tilsner et al. 2010; Tusnady and Simon 1998). The PMTV TGB3 (similar to TGB2) increases Pd SEL to allow movement of a GFP-sporamin fusion protein into neighboring cells (Haupt et al. 2005). The *Benyvirus* TGB3 protein is slightly smaller (≈ 15 kDa), has two transmembrane segments but lacks the N-terminal cysteine-rich region, and shares little sequence similarity with other TGB3 proteins (Lauber et al. 2001; Morozov and Solovyev 2003).

In contrast, the small (7 to 8 kDa) *Alpha-* and *Betaflexiviridae* TGB3 proteins contain a conserved CX₅GX₈C sequence and a single N-terminal transmembrane domain in which the C-terminal half of the protein is predicted to reside in the cytosol. Mutational analysis supports the notion of an N-terminal transmembrane domain and C-terminal cytosolic domain (Krishnamurthy et al. 2003; Morozov and Solovyev 2003). Mutations in the cytosolic domain of the PVX TGB3 protein impair virus movement but have been linked to enhanced protein turnover. Thus, mutational analysis has not been effective for identifying protein functions because they lead to greater protein instability, which itself may be deleterious to virus movement (Ju et al. 2008).

Interactions between TGB proteins.

The hordei-like TGB proteins of PSLV, BSMV, and PMTV participate in homologous interactions as determined by analytical techniques (i.e., yeast two-hybrid, in vitro translated products) and chemical cross-linking of infected extracts and affinity chromatography of TGB1 proteins expressed in yeast (Cowan et al. 2002; Leshchiner et al. 2006; Lim et al. 2008). The first two HELD motifs are responsible for TGB1 self-interactions (Lawrence and Jackson 2001a). Infectivity experiments with HELD mutants affecting TGB1 protein interactions also compromised virus cell-to-cell movement, thus demonstrating the biological relevance of these interactions (Lawrence and Jackson 2001a; Lim et al. 2009). In addition, BSMV TGB1 and TGB3 protein interactions have been observed by affinity chromatography of yeast extracts, and the residues required for the BSMV TGB2 and TGB3 protein interactions have been

mapped in the central hydrophilic loops of both proteins; however, TGB1 has not been shown to interact with TGB2 (Cowan et al. 2002; Lim et al. 2008). Heterologous interactions have been reported to occur between the PMTV and BSMV TGB2 and TGB3 proteins (Cowan et al. 2002; Lim et al. 2008) and PMTV TGB1 interacts with the PMTV minor capsid protein, CP-RT (Torrance et al. 2009).

In contrast, only a few studies of protein–protein interactions of *Potexvirus* TGB proteins have been reported. Yeast two-hybrid and other assays have confirmed TGB1 self interactions (Leshchiner et al. 2008) and TGB1:CP interactions (Samuels et al. 2007) (Verchot-Lubicz laboratory, *unpublished data*); physical associations of the TGB2 and TGB3 proteins have been more difficult to confirm, although homologous interac-

tions have been observed in vitro for TGB2 proteins (Hsu et al. 2008, 2009; Tseng et al. 2009).

Future studies should be directed to i) determine when and where primary TGB2–TGB3 protein interactions occur and the extent to which they affect membrane insertions of the TGB proteins and ii) assess sequence requirements for heterologous interactions of *Hordeivirus* TGB1, TGB2, and TGB3 proteins and the effects of disruptive mutations on cell biology and movement.

Diverse nature of TGB viral RNP movement complexes.

The role of the viral CP in virus cell-to-cell movement appears to be one of the most important differences between hordei-like and potex-like TGB proteins. In *Potexvirus* spp., the

Potex-like TGB: model based on TGB2 vesicles

Potex-like TGB: model of TGB3-driven transport

Hordei-like TGB: hordeivirus model

Hordei-like TGB: pomovirus model

- TGB1
- U TGB2
- J potex-like TGB3
- J hordei-like TGB3
- replicase
- vRNA
- virion
- virion modified by TGB1, or STP
- TGB1-vRNA-CP complex
- TGB1-vRNA complex

- remorin (REM)
- ▼ beta-1,3-glucanase
- ▼ beta-1,3-glucanase action
- TIP
- transport membrane vesicle
- actin filament
- ER tubule
- † † protein-protein interactions

CP is critical for cell-to-cell transport, but hordei-like TGB proteins can mediate cell-to-cell transport and, in some viruses, long-distance movement (Morozov and Solovyev 2003). Further studies have revealed substantial differences in the nature of viral transport RNP (the transport form of viral genomes) of these viruses.

Preparations of BSMV RNP complexes were isolated from infected tissues by sucrose density gradient fractionation (Brakke and Langenberg 1988) and found to contain the three positive-sense genomic RNAs, sgRNAs, and TGB1 proteins (Lim et al. 2008). No detectable amounts of other viral-encoded proteins or minus-sense RNAs that would suggest movement of a dsRNA replication complex were found (Donald et al. 1997; Jackson et al. 2009; Lim et al. 2008). Thus, complexes containing the two virus-specific components, plus-sense BSMV RNAs and TGB1 proteins, most likely represent the transport form of the genomic RNAs. Nevertheless, additional experimentation is required to determine whether the TGB1 protein, which shows sequence-nonspecific RNA binding in vitro, is able to interact specifically with BSMV RNAs in vivo, or whether TGB2 or TGB3 proteins have roles in RNP formation. One possibility is that TGB1-RNA complex formation could be initiated in compartmentalized replication sites that would permit nonspecific binding of highly concentrated and, possibly, nascent viral plus-sense RNAs. However, because the TGB1 protein binds ssRNAs in a nonspecific manner in vitro, as well as dsRNAs, the absence of BSMV minus-sense or dsRNAs in the RNP argues against this simple mechanism. Alternatively, nonsequence-specific plus-strand viral RNA complex formation might occur in vesicular bodies that are distinct from replication sites. For the *Pomovirus* PMTV, both TGB1 and TGB2 but not TGB3 proteins are reported to bind RNA in vitro in a sequence-nonspecific manner (Cowan et al. 2002). However, we do not know if they compose an RNP complex for cell-to-cell trafficking. TGB2 and TGB3 proteins are required to assist passage of TGB1 proteins to the Pd, although TGB2 and TGB3 proteins do not move between cells (Haupt et al. 2005;

Zamyatnin et al. 2004). It is possible that the nature of the RNP complex employed by *Pomovirus* spp. differs from the *Hordeivirus* RNP complex that is transported across Pd. Unfortunately, we do not yet know whether the *Hordeivirus* TGB2 protein binds RNA or is a component of the *Hordeivirus* RNP complex. We also are not certain if the *Pomovirus* TGB1 and TGB2 proteins form a single RNP complex in vivo. Comparative work is needed to know if the *Hordeivirus* and *Pomovirus* RNP complexes are similar or different. Furthermore, the nature of the *Benyvirus* RNP complex has yet to be explored.

For *Potexvirus* spp., RNP complexes are not easily purified because sedimenting virus particles interfere with their detection. Hence, the nature of the genome transport in vivo is obscure. However, two major transport models have been suggested. One model postulates that PVX can move through Pd in the form of virions. This view was supported by published electron micrographs showing fibrillar material resembling PVX virions embedded inside the Pd of PVX-infected leaves (SantaCruz et al. 1998). These structures reacted with antibodies capable of interaction only with PVX virus particles but not with isolated CP subunits, suggesting that these structures are virions (SantaCruz et al. 1998). Another model based on microinjection experiments and CP mutational analysis postulates that the *Potexvirus* transport form is a nonvirion TGB1-CP-RNA complex (Lough et al. 2000). One of the key experiments demonstrated that a WCIMV CP mutant, C-terminally truncated by five amino acids, was capable of virion formation but incapable of supporting viral cell-to-cell movement (Lough et al. 2000). The latter result suggests that virion formation per se is not sufficient to support cell-to-cell movement. This conclusion was further confirmed by studies of a similar C-terminally truncated PVX CP mutant which also appeared to be functional in virion formation but not in viral movement. Moreover, this mutant could be complemented by CP of unrelated viruses, such as *Potato virus A*, *Cocksfoot mottle virus*, and *Beet yellows virus*, and even by movement-deficient mutants of the TMV 30K MP (Fedorkin et al. 2000, 2001). These results sug-

Fig. 2. Four models of triple gene block (TGB) virus movement, summarizing the collective findings of each research group. The TGB proteins are designated p1, p2, and p3 in each panel. The endoplasmic reticulum (ER) is drawn as a double membrane structure around the nucleus and extending toward and across the plasmodesmata (Pd). Actin filaments are drawn as gray bars tightly associated with the ER. Host factors known to interact with the TGB proteins to regulate transport are illustrated where appropriate. The top two panels represent two possible transport routes for potex-like viruses. The top left panel shows viral replicase (brown spheres); TGB2 (U-shaped) and TGB3 (green bars) proteins are inserted along the ER. Replicase and TGB3 proteins move into membrane-bound bodies (MBB), which also contain virions. The model proposes that TGB1 protein interacts with these virions or virion-like particles to form a ribonucleoprotein complex that is transported to and across Pd. TGB1 protein interacts with remorin (REM) (orange sphere) at the cell wall. TGB1 also functions as a silencing suppressor protein and is phosphorylated by CK2. A second pathway has been proposed that employs TGB2 and TGB3. TGB2 induces novel vesicles to bud from the ER and these also contain TGB3 proteins. These vesicles associate with actin and move toward the Pd but we are not certain whether the vesicles mediate viral RNA cargo transport. We propose that TGB2 and TGB3 protein accumulation is regulated by the proteasome to limit cytotoxic effects and ER stress. The top right panel shows a model in which TGB3 proteins drive genome transport to peripheral membrane bodies (PMB) and across Pd. Here, TGB2 and TGB3 proteins colocalize in the ER. Callose synthesis is stimulated by virus infection and the accumulating callose molecules are deposited along the cell wall and in the plasmodesmata. The cell enzyme β -1,3-glucanase (red triangles) which hydrolyzes callose also interacts with TGB2 proteins via a cell protein TGB interacting protein (TIP) and is delivered to Pd using trafficking signals in TGB3 forming a complex with TGB2. TGB1-viral (v)RNA-coat protein (CP) complexes interact with TGB2 and TGB3 proteins along the ER, and their movement toward Pd is mediated by TGB3 proteins. The ribonucleoprotein complex moving through the Pd may consist of either TGB1-vRNA complexes or TGB1-vRNA-CP complexes; however, movement of either complex would require callose degradation at the Pd by β 1,3-glucanase. Question marks indicate putative protein-protein interactions that have not yet been confirmed experimentally. The lower left panel outlines the *Hordeivirus* transport route. Heterologous complexes of TGB2 and TGB3 proteins, illustrated as J- and U-shaped structures, are embedded in the ER. TGB1 forms ribonucleoprotein complexes by encapsidating genomic (g)RNAs and subgenomic (sg)RNAs in the cytoplasm, and these complexes then associate with membranes by forming binding interactions with the TGB3 moiety of the TGB2/TGB3:ER complex. TGB3 contains the Pd targeting signal that directs the complex to Pd, and such targeting is optimal when the relative ratios of TGB2:TGB3 are $\approx$ 10:1. The complexes are transported to Pd by unidentified components of the cellular transport machinery and the gRNAs and sgRNAs are deposited into adjacent cells. These RNAs then become available for translation in the recipient cell, and this process permits rapid expression of the sgRNA-encoded proteins required for movement and host defenses without the necessity of prior replication of the viral gRNAs. The lower right panel shows a transport model based on studies of *Potato mop-top virus* (*Pomovirus*) and there are similarities to the potex-like model drawn in the top right panel. In this case, the TGB2 and TGB3 proteins in the ER form motile granular structures that may represent vesicles similar to the potex-like TGB2 vesicles. TGB1-vRNA complexes bind to TGB2/TGB3 in the granular structures and are transported on the ER-actin network, and differ from the hordei-like model in this regard. However, like the hordei-like model, TGB3 proteins contain the Pd targeting signal that directs the complex to Pd, and Pd targeting is also optimal when the relative ratios of TGB2/TGB3 proteins are $\approx$ 10:1. TGB1-vRNA complexes move across the Pd, while the TGB2/TGB3 complex is absorbed into the plasma membrane. Some TGB2/TGB3 complexes are recovered from the plasma membrane via the endocytic pathway and return to the cell interior for additional rounds of transport.

gest that the C-terminal region of the potexviral CP harbors a function required for virus movement that can be uncoupled from the encapsidation function and complemented in trans. On the other hand, recent results support the concept that the N-terminal region of *Potexvirus* CP is also specifically involved in movement and can be separated into two distinct functional domains (Ozeki et al. 2009). These combined results can be explained either by the existence of an alternative transport complex, which could be fibrillar although nonvirion, or by an inability of virions formed by truncated CP to fulfill interactions required for transport. We should emphasize that the latter possibility does not contradict the virion model of a *Potexvirus* transport form.

The *Potexvirus* TGB1 protein interacts directly with CP (Ozeki et al. 2009) and encapsidates one end of the virion, namely the 5' terminus of the viral RNA, to induce energy-dependent conformational changes to virus particles in vitro (Atabekov et al. 2000; Lukashina et al. 2009). These so-called single-tailed particles (STP) consist of a helical rod-like head protein structure and an extended 3' RNA tail (Karpova et al. 2006; Kiselyova et al. 2003; Rodionova et al. 2003). On the one hand, these data are important in view of mutational analyses showing that a portion of the 5'-untranslated regions of the PVX and WCIMV genomes are essential for cell-to-cell movement of virions (Lough et al. 2006). On the other hand, it is possible that TGB1-modified virions or STP formed in vivo, where virion assembly is much more effective than in vitro, are the transport form of *Potexvirus* genome.

Comparisons of TGB-containing viruses have revealed an inverse correlation between the size or complexity of TGB1 protein structure and the necessity for viral CP in systemic infections of whole plants (Makarov et al. 2009). In the case of *Potexvirus* spp., where the TGB1 protein is smallest among the TGB viruses and lacks an NTD or ID, the CP is essential for both cell-to-cell and long-distance transport (Beck et al. 1991; Chapman et al. 1992; Spillane et al. 1997). In contrast, among viruses currently assigned to the hordei-like group, the CP is dispensable for cell-to-cell movement and, in these cases, the TGB1 protein and, possibly, other TGB proteins are believed to interact with genomic RNAs to form RNP movement complexes. However, some differences have been noted in CP requirements for long-distance movement that relate to N-terminal TGB1 variations. In BSMV and PSLV, which encode TGB1 proteins containing an extended NTD and ID domains, viruses containing dysfunctional CP mutations are able to establish systemic infections. However, an intermediate situation is found in BNYVV and *Peanut clump virus* (PCV), where the TGB1 protein is characterized by a shorter N-terminal extension region comprising the ID and a truncated NTD (Morozov and Solovyev 2003). In these cases, the CP is required for vascular transport as in the genus *Potexvirus* (Herzog et al. 1998; Schmitt et al. 1992; Tamada et al. 1996;). It has been proposed that the extended *Hordeivirus* NTD forms a natively unfolded domain that may function as an RNA chaperone to mediate phloem transport by stabilizing or protecting genomic RNAs (Makarov et al. 2009). Therefore, the more extended hordei-like TGB1 N-terminal regions may have functions similar to those of the potex-like virus CP in long-distance transport. However, PMTV provides a more complicated example of an intermediate TGB transport system. Although the PMTV TGB1 protein has an NTD similar in size to that of PCV (Makarov et al. 2009), the two PMTV genomic RNAs can move locally and systemically in the absence of the viral CP, presumably in the form of TGB1-formed RNP as in the *Hordeivirus* spp. (Savenkov et al. 2003). However, PMTV differs from all of the other hordei-like viruses in that the third genomic RNA encodes a CP with a novel read-through element

(CP-RT) that functions in long-distance movement of virus particles (Torrance et al. 2009). One end of this particle is most probably associated with the CP-RT and TGB1 to form a structure that resembles *Potexvirus* STP. Therefore, a hypothesis is that some viruses currently classified in the hordei-like group are able to exploit TGB RNP for localized movement of the viral genome but require structurally different virus-like particles related to those of the potex-like STP for long-distance transport. Hence, these localized movement phenotypes suggest the existence of more than one functional class of hordei-like TGB1 proteins and these differences, combined with the differences noted in the BNYVV TGB3 protein structure, prompt provisional suggestions outlined below for revision of the current hordei-like TGB classification.

Subcellular localizations of TGB proteins.

Crude cell fractionation and electron microscopy experiments initially demonstrated membrane and cell-wall associations of hordei-like TGB proteins during infection (Cowan et al. 2002; Donald and Jackson 1994; Erhardt et al. 2005; Niesbach-Klosgen et al. 1990). Immunogold electron microscopy of infected leaf tissues and fractionation studies also revealed that the *Potexvirus* TGB1 protein is localized in the cytoplasm and nuclei (Chang et al. 1997; Davies et al. 1993; Liou et al. 2000). However, the use of fluorescent protein reporter genes has provided higher resolution of TGB protein subcellular localization as well as temporal expression during virus movement (Erhardt et al. 2000; Lawrence and Jackson 2001a; Solovyev et al. 2000; Zamyatnin et al. 2002). For example, in one of the earlier studies, a GFP fusion of the 42-kDa TGB1 protein of an infectious BNYVV derivative was shown to require the functions of TGB2 and TGB3 proteins for Pd targeting (Erhardt et al. 2000). In addition, an infectious BSMV reporter virus GFP fused to the N-terminus of the TGB1 protein exhibited temporal expression of TGB1 protein and varied membrane associations as movement expanded radially from the centers of infection foci (Lawrence and Jackson 2001a). Similarly to BNYVV, BSMV GFP:TGB1 expression at the advancing edge of the foci was intense and paired punctate foci often extended across the walls into adjacent cells. Nearer the centers of the infection foci, and presumably in more advanced infections, greatly reduced levels of diffuse fluorescence were evident, in contrast to the uniform expression of another fluorescence virus reporter protein (γ b:GFP) involved in gene silencing. Protoplasts infected with the GFP:TGB1 reporter virus developed intense and diffuse GFP:TGB1 fluorescence at perinuclear membranes and formed punctate foci at the plasma membrane. These experiments clearly indicate that TGB1 undergoes complex membrane and cell-wall associations during different phases of infection (Lawrence and Jackson 2001a).

Several ectopic expression strategies with GFP:TGB protein fusions have been used to investigate TGB subcellular localization (Morozov and Solovyev 2003; Jackson et al. 2009). When TGB1 is expressed alone, it fails to associate with the Pd. One clear example is represented by PMTV TGB1. When introduced by microprojectile bombardment, GFP-TGB1 expression results in a diffuse fluorescence in the cytoplasm and nucleoplasm (Zamyatnin et al. 2004). GFP-fused BSMV TGB1 expressed independently of other viral proteins exhibits similar patterns in protoplasts (Lawrence and Jackson 2001a) and, in *Agrobacterium*-infiltrated cells, TGB1 is found in fluorescent bodies in the cytoplasm that appear to be adjacent to the cell wall; however, the fluorescence retracts along with the cytosol upon plasmolysis (Lim et al. 2009). Similar results were obtained with GFP-labeled PVX and BNYVV TGB1 expressed from viral genomic replicons (Erhardt et al. 2000; Samuels et al. 2007). As discussed below, although the require-

ments for localization of hordei-like and potex-like TGB1 proteins vary, it is generally accepted that TGB1 requires the presence of, or interactions with, TGB2 and TGB3 for appropriate localization.

Many experiments have established that *Potexvirus*, *Bennyvirus*, *Pomovirus*, and *Hordeivirus* TGB2 and TGB3 proteins associate with the ER and motile granules (Haupt et al. 2005; Ju et al. 2005; Lauber et al. 2001; Martelli et al. 2007; Mitra et al. 2003; Samuels et al. 2007; Solovyev et al. 2000). When GFP-TGB2 proteins are expressed in plant cells in the absence of other virus proteins, they localize primarily in polygonal ER networks, in ER-associated motile granules, and in endosome-like structures that appear at late stages of expression (Haupt et al. 2005; Ju et al. 2005; Lim et al. 2009; Mitra et al. 2003; Solovyev et al. 2000). Mutational analysis indicates that TGB2 membrane association is essential for cell-to-cell movement of most TGB-containing viruses (Lauber et al. 1998; Krishnamurthy et al. 2003; Mitra et al. 2003). Attempts to uncover the origins of the vesicles containing PVX TGB2 indicate that they are ER-derived and BiP-immunoreactive granular vesicles (Ju et al. 2005) (J. Verchot, R. Mitra, and D. Bamunusinghe, *unpublished data*) that are not obvious in healthy tissues (Bamunusinghe et al. 2009; Ju et al. 2005). Thus, the precise nature of the potex-like TGB2-specific ER-associated granules requires additional experimentation.

There are experimental data to support the hypothesis that the TGB3 proteins serve as a “driving force” for trafficking TGB1 proteins to the cell periphery for *Hordeivirus* spp. (BSMV and PSLV) and *Pomovirus* spp. (PMTV) (the hordei-like model is further described below). The BSMV TGB3 protein appears to be sufficient for Pd localization of its own TGB1 protein, although TGB1 targeting is most efficient in the presence of both TGB2 and TGB3 proteins. Both PMTV TGB2 and TGB3 proteins are needed for Pd transport of TGB1 proteins (Zamyatnin et al. 2004; Lim et al. 2008; Schepetilnikov et al. 2008). The *Bennyvirus* BNYVV also requires TGB2 and TGB3 to drive the TGB1 proteins to the periphery but the relationships of these proteins are not as well studied as for *Hordeivirus* and *Pomovirus* spp. (Erhardt et al. 2000). TGB3 also appears to drive trafficking of TGB2 to the periphery in PVX-infected cells (Schepetilnikov et al., 2005).

Different studies of TGB3 localization have given somewhat contradictory results for hordei-like and potex-like viruses. PSLV GFP-TGB3 ectopically expressed after particle bombardment of plant cells was first shown to localize in membrane bodies at the cell periphery (peripheral membrane bodies [PMB]) in close proximity to the cell wall (Solovyev et al. 2000) that requires the formation of homologous TGB3 complex interactions (E. A. Shemyakina, A. G. Solovyev, and S. Yu Morozov *unpublished data*). These TGB3-specific PMB contained an ER marker and, thus, could be considered to be derivatives of ER membranes (Zamyatnin et al. 2002). Whether these bodies represent nonspecific viral perturbations of cellular membranes or structures necessary for promoting virus egress is unknown. In contrast, expression of PMTV mRFP-TGB3 revealed an early association with ER and motile granules and later accumulated in a subpopulation of Pd (confirmed by co-localization with aniline-blue-stained callose) (Tilsner et al. 2010); peripheral bodies were not observed. In addition, PMTV TGB3 colocalizes with TGB2 in ER motile granules and targets TGB2 to the Pd (Haupt et al. 2005; Tilsner et al. 2010).

Different approaches using expression in transgenic plants, viral vector-infected plants, or *Agrobacterium*-infiltrated leaf tissue have provided a large body of evidence suggesting that BNYVV, PMTV, BSMV, and PSLV TGB3 proteins form paired structures on opposite sides of the cell wall that colocal-

ize with the Pd markers TMV P30 or callose (Gorshkova et al. 2003; Erhardt et al. 2005; Haupt et al. 2005; Schepetilnikov et al. 2008; Lim et al. 2009). However, analyses of the TGB3-specific associations following cell plasmolysis have provided somewhat different results for transgenically expressed PSLV TGB3 and ectopically expressed BSMV TGB3 from *Agrobacterium* or PMTV TGB3 after bombardment. Here, the transgenically expressed PSLV TGB3 protein retracted from the cell wall upon plasmolysis, whereas a portion of the transiently expressed BSMV TGB3 and PMTV TGB3 protein remained associated with the cell wall (Gorshkova et al. 2003; Lim et al. 2009; Tilsner et al. 2010). Regardless of these differences that need additional comparisons, all of the coexpression studies with BNYVV, PMTV, BSMV, and PSLV are in agreement that TGB3 has a major role in targeting TGB1 and TGB2 proteins to peripheral bodies or the cell wall in the vicinity of Pd (Solovyev et al. 2000; Morozov and Solovyev 2003; Zamyatnin et al. 2004; Jackson et al. 2009). Additional evidence suggests that TGB3 interactions with TGB1 and TGB2 proteins are required for TGB1 localization and the ability of hordei-like viruses to function in subcellular targeting and in cell-to-cell movement (Cowan et al. 2002; Lim et al. 2009; Tilsner et al. 2010). Mutagenesis studies showed that the two C-terminal Lys and Arg residues were involved in retaining BSMV TGB3 proteins at the cell wall (Lim et al. 2009). In addition, the conserved TGB3 peptide motif YQDLN is essential for cell wall transport (Solovyev et al. 2000; Schepetilnikov et al. 2008). Alteration of the YQDLN motif in the PMTV TGB3 protein to 3GQDGN also abolishes ER labeling, motile granule formation, and Pd targeting (Haupt et al. 2005), and a single amino acid exchange G for Y in the motif abolished virus cell-to-cell movement (Tilsner et al. 2010).

For *Bennyvirus* spp., advances were made by adding C-terminal hemagglutinin tags to TGB2 or TGB3 in the BNYVV genome, which allowed the study of their subcellular localization during virus infection (Erhardt et al. 2005). Interestingly, membrane-rich peripheral bodies (MRPB) derived from disorganized ER were found near cell walls and accumulated TGB1, TGB2, and TGB3 proteins. Pd and MRPB-targeting were dependant on expression of the entire TGB cluster (Erhardt et al. 2005). Although we do not yet know if these MRPB represent sites for assembly of viral genomes into a transport complex prior to Pd entry, or the destination of proteins after the genomes have been transferred into the pore, the MRPB represents structures unique to BNYVV. Thus, future research could uncover events in the intracellular transport pathway that appear unique to BNYVV and are not yet associated with viruses in the *Virgaviridae* family.

For *Potexvirus* spp., TGB3 localization reveals striking differences from the patterns found in hordei-like TGB3 proteins. PVX GFP-TGB3 associates with the polygonal network of ER tubules (Krishnamurthy et al. 2002). PVX TGB3-GFP also associates with the motile granules containing CFP-TGB2, although their movement toward the peripheral bodies or Pd was not tested in the same study to know whether these granules are involved in relocation to the cell-wall domain (Samuels et al. 2007). On the other hand, in the presence of the native TGB3, PVX GFP-TGB3 relocates to peripheral bodies containing an ER marker closely resembling the PMB formed by hordei-like TGB3 proteins (Schepetilnikov et al. 2005). Thus, there are two points of view on PVX TGB3 protein localization. One view implies that wild-type PVX TGB3 is localized to the ER tubules and TGB2-containing vesicles. Another interpretation is that the GFP-fused TGB3 subcellular targeting is affected by the marker protein fusion, and that coexpression with wild-type TGB3 redirects the GFP-TGB3 to PMB that are authentic sites of PVX TGB3 localization. Supporting evidence for the

former notion is that a C-terminal GFP fusion of TGB3 also localizes to the ER (Ju et al. 2008; Bamunusinghe et al. 2009), and support for a PMB site of localization is that GFP-TGB3 is deficient in supporting viral movement (Schepetilnikov et al. 2005). These two contradictory views on PVX TGB3 localization give rise to different models of PVX TGB-mediated transport (Fig. 2).

In the future, a study of mutations that affect protein–protein interactions might provide more direct information as to the roles of individual TGB proteins in localization of complexes from infectious entities.

TGB interactions with host proteins.

Only a small amount of information has been published on the interactions of hordei-like TGB proteins with plant host factors. One report has shown that the PMTV TGB2 protein interacts in vitro with a tobacco DnaJ protein belonging to the receptor-mediated endocytosis-8 family of endosomal trafficking proteins, and TGB2 colocalizes in vesicles with Ara7 (AtrabF2b), a Rab GTPase involved in endosome trafficking (Haupt et al. 2005). Another report indicates that the PSLV TGB3 protein partially colocalizes with the cellular At-4/1 protein, which is capable of autonomous trafficking through the Pd (Paape et al. 2006). Clearly, an important future goal is to focus additional work on identification of host–protein interactions and the functional requirements for such interactions.

Interactions of *Potexvirus* TGB proteins with several plant host factors have been described. The protein kinase CK2 can phosphorylate PVX TGB1 as well as *Tomato mosaic virus* P30 movement protein (Matsushita et al. 2003; Lee 2008; Modena et al. 2008). More recent evidence points to protein phosphorylation regulating PVX movement (A. Zelada, *personal communication*), although the direct impact of protein modification on assembly of the RNP complex or Pd gating has not been reported. PVX TGB1 was found to interact with remorin (REM), a protein located in the cytosolic leaflet of plasma membrane microdomains (lipid rafts) and in clusters at Pd. REM can be phosphorylated in the presence of oligogalacturonides (Raffaele et al. 2009). REM overexpression inhibits the movement of PVX through the Pd. This work raises the hypothesis that membrane rafts play an important role in macromolecular trafficking. The authors speculate that REM binding to the TGB1 proteins within membrane rafts could effectively titrate out TGB1 protein activity and prevent it from carrying out its role in virus movement (Raffaele et al. 2009). A yeast two-hybrid screen has also identified novel PVX TGB2 interacting plant proteins (TIP1, TIP2, and TIP3) that interact with β -1,3-glucanase to regulate callose degradation and the SEL of the Pd (Fridborg et al. 2003). Hence, these interactions provide a plausible mechanism for virus movement (Bucher et al. 2001; Epel 2009a) and can also be broadly integrated into a global movement model that is compatible with the postulated role of callose degradation in promoting TMV cell-to-cell trafficking (Epel 2009a).

Possible links between virus replication and TGB-mediated transport.

Another important area that merits more attention is the role that TGB proteins may play in initiating complexes with replicated progeny RNAs and whether this occurs at sites of replication. For example, in the presence of replicating viral RNAs, a portion of BSMV TGB2 proteins has been found to accumulate at chloroplasts (Torrance et al. 2006), where outer membrane vesicles form the putative sites of BSMV RNA replication (Lin and Langenberg 1985). The RNA-binding activities reported for PMTV TGB2 also suggest that TGB2 proteins

may have an important role in RNP complex formation at diverse sites of viral replication; mRFP-TGB2 fusions localize to chloroplasts with CP, genomic, and minus-strand RNA (G. Cowan and L. Torrance, *unpublished*). Furthermore, the abilities of TGB1 proteins to bind dsRNAs combined with their associated RNA helicase unwinding activities are compatible with a model whereby subsets of replicating RNAs form TGB1-containing RNP complexes. In the case of BSMV, one possibility is that, during the early stages of transport, TGB2 proteins localize to the chloroplast sites of replication, bind replicating RNA complexes, and form heterologous associations with TGB3 proteins. Due to its association with TGB3, TGB2 could interact with TGB1 to mediate associations with dsRNA replicative intermediates and unwind these intermediates to form nascent RNP derivatives. At intermediate stages in the process, the RNP complex could be transported to peripheral vesicles by currently undefined elements of the cellular machinery as a prelude to transit through the desmotubule and release of the cargo RNA into adjacent cells. After these activities are completed, TGB2, TGB3, and possibly TGB1 proteins could be recycled back to replication sites via the endocytic pathway (Haupt et al. 2005).

Although, in some viruses, the TGB module can be functionally complemented by substitution with 30K-like movement proteins, suggesting independence of replication and movement functions as previously mentioned, these recombinant viruses are usually not as “fit” as wild-type isolates. Another possibility is that TGB proteins enhance pathogenicity and that association of TGB proteins at sites of replication reflects a role in the temporal or spatial regulation of the competing processes of virus replication from translation and transport, perhaps by separating the RNA template in membranous structures or chloroplast invaginations for replication and production of particles later in the infection cycle.

PVX genomic RNA mainly associates with membrane-bound factories from the perinuclear ER that contains replicase and TGB1 proteins (Tilsner et al. 2009). The *Potexvirus* TGB3 protein also associates with the cortical ER network as well as membrane-bound bodies (MBB) containing PVX replicase, virions, and TGB1 proteins (Krishnamurthy et al. 2003; Ju et al. 2005; Samuels et al. 2007; Bamunusinghe et al. 2009), whereas TGB2 proteins accumulate in post-ER vesicles. These vesicles are essential for cell-to-cell movement and align to actin filaments (Ju et al. 2005, 2007; Tseng et al. 2009). The BaMV TGB2 protein, similar to *Pomovirus* TGB2 protein, binds viral RNA; it is not known whether it forms part of the RNP complex with TGB1 and CP but there are speculations that the TGB2 protein facilitates intracellular delivery of viral RNA to Pd (Hsu et al. 2009). Although we do not know whether these MBB contain viral RNA templates involved in replication, these structures are seen early in virus infection and lead us to propose that they function as sites for RNA translation, synthesis, and encapsidation (Bamunusinghe et al. 2009). The possible roles of TGB3 inside these structures are speculative, although it is worth noting that one potential TGB3 function is to promote viral RNA export from the MBB to the cell periphery.

Potex-like TGB transport models.

In accordance with the two points of view concerning PVX TGB3 protein subcellular localization, we are proposing two models for *Potexvirus* TGB-mediated transport (Fig. 2). The first model is based on TGB3 protein association with the cortical ER. GFP-TGB3 fusions associate broadly with the ER whereas PVX TGB2 and replicase proteins are each restricted to separate subcompartments containing ER membranes. Specifically, TGB3 is seen in the ER network, MBB containing

PVX replicase, and a separate population of vesicles containing TGB2 (Bamunusinghe et al. 2009; Ju et al. 2005; Krishnamurthy et al. 2003; Samuels et al. 2007). Given that early studies revealed that deleting TGB3 from the viral genome does not hamper virus replication, TGB3 is not likely to function as the membrane anchor for the PVX replicase (Beck et al. 1991; Verchot et al. 1998). Typical membrane-anchoring proteins are essential and knockout mutations would eliminate virus replication. Perhaps ER-remodeling during virus infection enables TGB3 to be captured into MBB as infection progresses. On the other hand, TGB3 binding to the ER is essential for virus intercellular transport (Krishnamurthy et al. 2003), although there has been little progress toward clarifying the role for the ER in modulating PVX cell-to-cell movement.

The TGB2 protein accumulates in post-ER vesicles that are either novel structures or might be related to the COPI or COPII machinery. These vesicles align with actin filaments, and mutational analysis indicates that they are essential for cell-to-cell movement (Ju et al. 2005, 2007; Tseng et al. 2009). Thus, a membrane-mediated route for intracellular transport entails TGB2-induced vesicles carrying infectious material to the Pd for cell-to-cell transport (Fig. 2) (Hsu et al. 2009). The TGB2 central ER luminal domain has a long segment of conserved amino acids. Substitution of any one of these residues with a nonconserved residue is sufficient to cause PVX-derived GFP-TGB2 fusions to accumulate, mainly in the ER and in ER protrusions, while depleting granular vesicles in which they also associate (Ju et al. 2008). Mutations depleting granular vesicles also inhibit PVX cell-to-cell movement. Furthermore, two cysteine residues, Cys-109 and Cys-112, at the *Potexvirus* BaMV TGB2 C-terminal tail, which appears to be exposed on the outer surface of the membrane, and ER-derived vesicles are important for protein function (Ju et al. 2008; Tseng et al. 2009).

There is evidence that TGB3 and TGB2 proteins colocalize in vesicles (Samuels et al. 2007), and it can be proposed that transition of TGB3 from the ER and MBB into vesicles may be a factor in transferring viral RNA or TGB1-vRNA-CP complexes to transport vesicles populated by TGB2 proteins. Investigations thus far have failed to determine whether these vesicles transport other viral proteins or infectious material toward the Pd (Bamunusinghe et al. 2009) (*unpublished data*). One option is that infectious material is carried along the outer surface of the vesicles, rather than in the interior, although this possibility would be complicated to analyze. Therefore, further research is needed to identify the viral components of these vesicles and their exact role in trafficking virus to the Pd.

An alternative model posits that the PVX TGB3 protein localizes to sites that are Pd-associated PMB. If this is the case, TGB3 of *Potexvirus* spp. may act similarly to their counterparts in hordei-like viruses by directing TGB2- and TGB1-containing RNP to Pd-associated PMB. There is no experimental evidence thus far to implicate TGB1 protein targeting to Pd by the combined activities of the *Potexvirus* TGB2 and TGB3 proteins; however, PVX GFP-TGB2 is directed to peripheral bodies by nonfused TGB3 (Solovyev et al. 2000; Schepetilnikov et al. 2005). Because TGB2 is able to both interact with the TIP host proteins to affect β -1,3-glucanase activity (Fridborg et al. 2003) and increase the Pd SEL (Tamai and Meshi 2001), one can presume that TGB3-directed targeting of TGB2 to Pd-associated sites is required for *Potexvirus* Pd gating. This alternative model predicts that potex-like TGB3 contains specific PMB targeting signals and is transported intracellularly by an unconventional COPII-independent mechanism (Schepetilnikov et al. 2005). The mechanism of delivery of *Potexvirus* transport RNP to Pd probably occurs through virions modified by interactions with TGB1, and

further trafficking through the gated Pd may be assisted by TGB2 because this protein has RNA-binding properties (Hsu et al. 2009).

The model of TGB3 driving transport to the cell periphery was recently supported by fluorescence-imaging studies that examined the localization, trafficking, and interactions of BaMV TGB2 and TGB3 proteins in yeast cells. TGB2 proteins, when expressed in the absence of TGB3, accumulate in ER structures, whereas TGB3 proteins expressed alone accumulate in PMB (Lee et al. 2010). Detailed studies in yeast cells revealed that TGB2 and TGB3 proteins interact in the ER, giving a stoichiometric complex, and further assemble into structures capable of moving to the periphery of the cell, where they form PMB (Lee et al. 2010). As shown by a number of approaches, the transport of these multimeric complexes to the cell periphery requires neither the cytoskeleton nor the Golgi-dependent secretory pathway in yeast (Lee et al. 2010). However, the actin and microtubule networks do not play a role in regulating movement and organization of the ER network in yeast as they do in higher plants and other eukaryotes. These studies of BaMV in yeast are contrasted by experiments in which PVX GFP-TGB2 proteins containing vesicles were visualized along actin filaments in plant cells, and treatment with latrunculin B caused dispersal of vesicles (Ju et al. 2005). Moreover, a recent publication demonstrates that intact microfilaments are important for PVX cell-to-cell movement in *Nicotiana benthamiana* (Harries et al. 2009). Therefore, the BaMV data obtained in yeast require verification in plants. Because the relationship of the cytoskeleton with the ER is different in yeast than in plant cells, these outcomes further highlight the need to clarify the relative roles of the actin and ER networks in the intercellular transport of *Potexvirus* spp. and other TGB-containing viruses in plant cells. Very recent data suggest a model whereby dynamic three-way interactions between ER, F-actin, and myosins determine the architecture and movement patterns of ER strands (Ueda et al. 2010). For TMV, it was demonstrated that the 30K movement protein can move from cell to cell by diffusion in the ER component of the Pd (Guenoune-Gelbart et al. 2008). Researchers proposed that the ER potentially serves as a conduit for diffusional movement of virus RNAs associated directly or indirectly with viral MPs which are anchored in the ER (Guenoune-Gelbart et al. 2008; Epel 2009b).

Model for hordei-like TGB-mediated transport.

Hordei-like TGB protein functions require a number of protein-protein interactions for virus transport into adjacent cells. In the presence of BSMV, PSLV, and PMTV TGB3, the cognate GFP-TGB2 proteins are relocalized from cytoplasmic membranes to cell-wall structures (Solovyev et al. 2000; Zamyatnin et al. 2004; Lim et al. 2009). TGB3-directed subcellular targeting of TGB2 could be easily explained by formation of specific complexes containing both proteins, and recent observations of heterologous binding of the two proteins supports this hypothesis. Surprisingly, earlier studies using heterologous TGB proteins suggested that TGB3-directed membrane-embedded targeting might involve a sequence-independent mechanism (Solovyev et al. 2000; Zamyatnin et al. 2002). However, more recent experiments employing affinity chromatography and yeast two-hybrid interactions have shown that hordei-like TGB2 and TGB3 interactions occur (Cowan et al. 2002; Lim et al. 2008). Moreover, mutations in both proteins affecting amino acid residues that disrupted TGB2-TGB3 protein interactions also blocked TGB3-dependent TGB2 targeting to cell walls and, when introduced in a BSMV infectious cDNA clone, these mutations resulted in a loss of cell-to-cell movement (Lim et al. 2009). These data provide support for the

hypothesis that direct interactions of TGB2 and TGB3 are required for movement activities of viruses encoding hordei-like TGB modules (Jackson et al. 2009).

Coexpression of BSMV GFP-TGB1, which is located in cytoplasmic bodies when expressed individually, with BSMV TGB2 and TGB3 resulted in localization of a proportion of the fluorescent protein to discrete cell-wall-associated foci. Importantly, coexpression of GFP-TGB1 with only TGB3 also gave rise to small but detectable amounts of such TGB1 protein-containing cell-wall-embedded foci, whereas coexpression of GFP-TGB1 with TGB2 did not result in cell-wall association of TGB1 (Lim et al. 2009). These findings are in good agreement with affinity chromatography data showing that BSMV TGB1 is able to interact with TGB3 but not TGB2 proteins *in vitro* (Lim et al. 2008). An accessory role of TGB2 in TGB3-directed TGB1 protein targeting to the cell wall is further confirmed by mutations that abrogate TGB2–TGB3 interactions. In these experiments, if wild-type TGB2 and a mutant TGB3 or a mutant TGB2 and wild-type TGB3 were coexpressed, most but not all of the TGB1 retracted from the cell wall upon plasmolysis, whereas the combination of wild-type TGB2 and TGB3 resulted in larger amounts of TGB1 protein localization at the cell wall after plasmolysis (Lim et al. 2009). Thus, TGB3 appears to be sufficient for the cell-wall localization of BSMV TGB1 proteins, although cell-wall targeting of the TGB1 protein is more efficient in the presence of TGB2 (Lim et al. 2009).

The BSMV and PSLV *Hordeivirus* data are in good agreement with conclusions from PMTV TGB1 protein targeting obtained with a different technical approach. Using biolistic bombardment, the GFP-fused PMTV TGB1, when expressed alone, failed to localize to the cell wall but, in the presence of both TGB2 and TGB3 proteins, GFP-TGB1 localized to tiny cell-wall-embedded structures that presumably represent Pd, and fluorescence moved into neighboring cells (Zamyatnin et al. 2004). Importantly, unlike BSMV, both TGB2 and TGB3 proteins are required for cell-wall targeting of PMTV TGB1. Site-specific mutations of PMTV TGB1 proteins revealed that a mutation in the conserved motif I of the NTPase/helicase domain had no discernable effects on targeting to the cell wall (Zamyatnin et al. 2004). Similarly, mutations in the BSMV TGB1 motif I failed to affect localization to plasmolyzed cell-wall-associated structures in the presence of both TGB2 and TGB3 proteins (Lim et al. 2009). Because this motif is involved in TGB1 NTPase activity, self interactions, and RNA binding (Leshchiner et al. 2006; Lim et al. 2008), these protein functions are apparently unnecessary for TGB2- and TGB3-mediated targeting to cell-wall sites. These data are sufficient to conclude that TGB1 has a passive cargo role in its targeting to cell-wall sites by TGB2 and TGB3 proteins. It should also be noted that associations of BNYYV TGB1 at the cell periphery require TGB2 and TGB3; however the relationships of these proteins are not as well studied as for *Hordeivirus* and *Pomovirus* spp. (Erhardt et al. 2000).

Transport of hordei-like TGB3 to peripheral bodies and Pd requires specific TGB3 residues. Both PSLV and PMTV TGB3 localization experiments have provided evidence for a C-terminal requirement for targeting to the cell periphery (Schepetilnikov et al. 2008; Tilsner et al. 2010). Deletions in the C-terminal hydrophobic transmembrane domain inhibited Pd targeting of PSLV, indicating a composite Pd-targeting signal (Solovyev et al. 2000). Furthermore, PMTV TGB3 was shown to encode a bipartite signal comprising the conserved luminal motif YQDLN and the C-terminal transmembrane domain (Tilsner et al. 2010). The conserved peptide motif YQDLN was essential for cell-wall transport of PSLV TGB3 proteins (Schepetilnikov et al. 2008; Solovyev et al. 2000). Alteration of the YQDLN motif in PMTV TGB3 to GQDGN also

abolished ER labeling, motile granule formation, and Pd targeting of the mutant mRFP-TGB3 protein (Haupt et al. 2005), and incorporation of a G for Y mutation into an infectious reporter virus inhibited cell-to-cell movement (Tilsner et al. 2010). Site-specific mutagenesis experiments have shown that the two C-terminal amino acid residues are required for retention at the cell wall of both BSMV and PMTV TGB3 proteins (Lim et al. 2009; Tilsner et al. 2010).

Direct evidence is not available to identify subcellular transport pathways functioning in transit of the hordei-like TGB proteins. However, the Morozov group has recently reported that suppression of the formation of ER-derived COPII-coated vesicles by a dominant-negative mutant of the Sar1 small GTPase has no detectable effects on PSLV TGB3 trafficking to peripheral bodies (Schepetilnikov et al. 2008). Similarly, COPII-independent transport appears not to be involved in the targeting of PVX TGB3 to cell-wall-associated sites (Schepetilnikov et al. 2005). Moreover, disintegration of Golgi stacks by brefeldin A treatment of leaves expressing PSLV GFP-TGB3 had no obvious effect on TGB3 localization to cell-wall-associated peripheral bodies (Schepetilnikov et al. 2008). In addition, chemical inhibitors of microtubule and microfilament stability failed to prevent PSLV TGB3 transport to cell-wall-associated structures (Schepetilnikov et al. 2008). Collectively, these observations have led to the hypothesis that *Hordeivirus* TGB3 intracellular transport does not require the ER-Golgi secretory pathway or the cell cytoskeleton and, instead, involves an unconventional mechanism connected, most probably, to diffusion in a lipid bilayer (Schepetilnikov et al. 2008). This hypothesis, however, should be treated with caution until more definitive positive genetic findings can be obtained (Jackson et al. 2009).

In a different approach, PMTV mRFP-TGB2 and mRFP-TGB3 fusions were expressed in epidermal cells of transgenic plants in which the ER, microfilaments, or microtubules were labeled with GFP (Haupt et al. 2005). In this case, chemical inhibitors showed that movement of ER-associated motile granules containing the TGB proteins was completely inhibited by treatment with latrunculin B, which destroys actin filaments, but not by oryzalin, which disrupts microtubules. Furthermore, the mRFP-TGB2 or TGB3 did not colocalize with Golgi bodies and treatment with brefeldin A, which causes reabsorption of Golgi to the ER, did not affect the localizations of TGB. Thus, these data lead to the conclusion that TGB-mediated transport of PMTV to the cell periphery occurs in association with the ER-actin network, and that actin-mediated vesicular trafficking occurs in motile granular structures containing TGB2 and TGB3 (Haupt et al. 2005). Thus, the current cellular model for PMTV movement is that TGB3 and TGB2 proteins interact to facilitate TGB1-RNA transport. The TGB-RNA complexes reach the cell wall by trafficking along the ER using an actin-based motility system. When the complexes reach the Pd, they become anchored through interactions with components of the desmotubule and facilitate transport of viral RNAs through the pore. Another unique aspect of the current model is that TGB2:TGB3 complexes associate with the endosomal network, possibly to return to sites of virus replication where they interact with TGB1 and acquire viral RNP for further rounds of transport (Fig. 2).

Whatever the molecular mechanism of transport to cell-wall-associated structures, both hordei-like models are in agreement that the ability of the TGB3 protein to enter the translocation pathway is determined by specific signals in the TGB3 protein sequence. Moreover, mutational analyses of BSMV, PMTV, and PSLV TGB3 have revealed critical requirements of TGB3 in subcellular transport and localization at or near Pd (Lim et al. 2009; Schepetilnikov et al. 2008; Tilsner et al. 2010).

Intracellular trafficking of *Benyvirus* spp. has not been studied using these techniques. However, ER-derived MRPB were observed close to the cell wall in thin sections of tissue when all three BNYVV TGB proteins were expressed together but not when expressed individually or when virus cell-to-cell movement was mediated by the TMV 30K movement protein (Erhardt et al. 2005). The MRPB may represent sites of replication or assembly of movement complexes. However, it is unlikely that BNYVV TGB proteins represent replicase anchors because RNA1 replicates independently in protoplasts (Gilmer et al. 1992) and, more importantly, in BNYVV mutants, where the TGB cluster is replaced by the TMV 30K movement protein, is still able to move from cell to cell. Moreover, BNYVV cell-to-cell movement is inhibited by overexpression of *Benyvirus* TGB3 and TGB3 from the family *Virgaviridae* but not *Potexvirus* TGB3 (Lauber et al. 2005), which indicates that members of *Virgaviridae* and *Benyvirus* spp. utilize a common TGB3-mediated mechanism for cell-to-cell movement.

Summary and future perspectives.

The models presented for each virus reflect the experimental data that support several possible mechanisms for viruses to achieve intercellular transport using the TGB module. The experimental data indicate clear differences between potex- and hordei-like TGB subcellular interactions and movement mechanisms, and current evidence also indicates some differences between *Pomovirus* and *Hordeivirus* spp. There is insufficient information to determine whether *Benyvirus* proteins are functionally similar to members of the family *Virgaviridae*. Additional research is needed to further understand the nature of the TGB interactions and the extent to which these interactions are conserved among other members of *Flexiviridae* and *Virgaviridae* families and *Benyvirus* spp. In truth, the biological functions of TGB proteins belonging to the *Potexvirus* genus are among the only members of *Flexiviridae* that have been extensively studied thus far. There have been relatively few studies of *Carlavirus*, *Allexivirus*, *Mandarivirus*, and *Foveavirus* spp. to know whether the potex-like TGB-based transport system is a model that can be readily applied to these viruses. Considering the broad host range of these plant viruses, it is possible that individual members have adopted diverse strategies to ensure successful infection as they coevolve with their hosts. In fact most members of *Potexvirus*, *Carlavirus*, and *Allexivirus* infect herbaceous plants while *Foveavirus* and *Mandarivirus* spp. infect woody hosts. It is reasonable to consider that there may be host-specific virus–host interactions that have evolved to enable spread into tissues that are found in woody hosts which are not conserved with viruses infecting herbaceous species. Further comparative analysis of members belonging to other virus genera within *Flexiviridae* is needed to determine the extent to which these viruses conform to a potex-like TGB-based transport system. Similarly, some of the differences observed between the *Hordeivirus* and *Pomovirus* spp. could be attributed to the differences in virus host range, with BSMV and PSLV naturally infecting graminaceous hosts versus dicots for the *Pomovirus* spp. In addition, PMTV is soilborne, being transmitted by *Spongospora subterranea*, in contrast to the seedborne BSMV.

Furthermore, the role of membranes in virus movement to Pd could be virus specific. Lessons learned from *Picornavirus* spp. are that phylogeny can predict membrane associations but not necessarily the membranous routes of protein trafficking. Importantly, members of the *Cardovirus*, *Rhinovirus*, *Parechovirus*, and *Enterovirus* genera (within the family *Picornaviridae*) have different sensitivities to brefeldin A and requirements for COPI machinery to support viral complexes (Armer et al. 2008; Gazina et al. 2002; Wessels et al. 2006). Thus, the

relatedness of viruses within a family does not necessarily equate with the specific membrane subcompartments needed to support protein complexes. Among TGB-containing viruses, broad statements have been made describing TGB2 and TGB3 as central elements in guiding RNP complexes from intracellular locations to Pd. Given that each TGB-containing virus replicates on different membranes, it is possible to consider that the path of virus trafficking within the cell is unique and, therefore, the nature of the TGB2 and TGB3 membrane associations for trafficking to Pd may vary for each virus. There is not yet enough information available for us to conclude whether membrane associations identified for one virus are conserved across all TGB-containing viruses, among hordei-like or potex-like proteins, or even within genera. Future work will require comparative analysis of several virus members within each genus to know the extent to which various membrane requirements for virus movement are conserved.

Interactions of TGB viruses with their various hosts represent another area that could profit from further investigation. Most TGB-containing viruses, with the notable exceptions of *Lychnis ringspot virus* and PSLV, have been isolated from crop species or plants of agricultural importance. However, these viruses can collectively infect a wide range of experimental hosts. For example, most TGB viruses, including monocot-infecting BaMV and BSMV, are able to infect the model host *N. benthamiana* and other tobacco species. Because of its susceptibility to these viruses and its convenience as a model host, *N. benthamiana* has been used extensively for analysis of TGB functions. Although the efficiency of movement of TGB-containing viruses in their native hosts versus *N. benthamiana* has not been rigorously compared, a consensus is that TGB proteins facilitate movement by interacting with a conserved set of host factors dedicated to trafficking of host macromolecules. Nevertheless, attempts to probe functions of TGB proteins in different hosts suggested that the TGB proteins have varied functions in different hosts. In the first attempts with PVX, localization of TGB proteins was compared in several *Solanaceous* species (Howard et al. 2004; Krishnamurthy et al. 2003). These findings indicate that *N. benthamiana* may be more permissive than related hosts for movement of TGB2 and TGB3, because cell-to-cell movement of reporter gene fusions was observed in *N. benthamiana* but was constrained to single cells of *N. tabacum*, *N. clevelandii*, *Lycopersicon esculentum*, *Chenopodium quinoa*, and *Gomphrena globosa*. In a subsequent study, Howard and associates (2004) observed that injected CP reporter fusions had movement patterns similar to those of TGB2 and TGB3 in leaf cells. In contrast, GFP:TGB1 was found to move from injected cells to adjacent cells of each of the tobacco species and tomato but GFP:TGB1 mutants with inactivated ATPase were able to move only from injected *N. benthamiana* cells. In the case of BSMV, similarities were observed in early movement events in barley and oat but systemic movement in oat was delayed by several days compared with the natural barley host. In addition, the timing of systemic oat infections appeared to be similar to that of *N. benthamiana*, in which GFP fluorescence emerged from the veins at 8 to 10 days postinoculation. These results indicate that TGB interactions may vary in different hosts, and that host-specific variations in virus movement may depend on such interactions.

Variations between dicot and monocot leaf architecture also have been postulated to have substantial effects on early stages of BSMV movement (Lawrence and Jackson 2001b). In *N. benthamiana* and *C. amaranticolor*, primary infection foci arising from infections of single cells were easily detected by 24 h postinoculation (hpi) in leaves inoculated with BSMV containing GFP fusions to γ B. Rapidly expanding BSMV foci

consisting of several cells were evident by 48 hpi, and radial spread of the foci continued through a large number of mesophyll cells until the virus encountered reticulate veins. In contrast, extensive observations of inoculated barley and oat leaves failed to reveal singly infected cells or cell foci for the first 48 hpi. Nevertheless, by 72 hpi, vascular invasion of the cereal hosts was evident due to rapid virus spread through the highly concentrated parallel vascular bundles. By 96 hpi, the virus had begun to emerge at several locations along the length of the vascular bundles of upper uninoculated leaves of barley and, by 120 h, when systemic mosaic symptoms became evident, most of the uninoculated leaf mesophyll cells exhibited fluorescence. These results are compatible with a model whereby the early events in local movement are impacted by host vasculature. During BSMV infection of barley, long-distance transport appears to be accelerated by the dense parallel vasculature, whereas virus replicating in dicots with a reticulate vasculature must traverse numerous cells before encountering the vascular system. In addition, differences in rates of long-distance movement noted between barley and oat suggest that, even though common host factors facilitating local and vascular movement may be shared among monocots and dicots, variations of the interactions of the TGB proteins with these host factors have substantial roles on rates of systemic spread and disease development. A major challenge for the future will be to identify the nature of these interactions and account for their effects on infection processes.

To summarize, further research is needed to understand the interactions of the TGB proteins with host proteins and membrane components, which are essential for promoting virus cell-to-cell movement and may reveal additional supplementary functions. In addition, the dynamic temporal nature of the process, where interactions may change during the infection cycle, should also be considered. Much progress has been made using confocal and electron microscopic imaging, and some of the hypotheses that have been developed based on these data need to be supported by further genetic and biochemical studies. Imaging will not differentiate between other possible functional roles of the TGB during the infection cycle, which may have more to do with biology of the particular virus such as specific virus–host or virus–vector interactions or in contributing to the spatial separation or regulation of the processes of replication and translation.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

International Committee on Taxonomy of Viruses website:
www.ictvonline.org/virusTaxonomy.asp