

REVIEW

Molecular biology of potexviruses: recent advances

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Recent advances in potexvirus research have produced new models describing virus replication, cell-to-cell movement, encapsidation, *R* gene-mediated resistance and gene silencing. Interactions between distant RNA elements are a central theme in potexvirus replication. The 5' non-translated region (NTR) regulates genomic and subgenomic RNA synthesis and encapsidation, as well as virus plasmodesmal transport. The 3' NTR regulates both plus- and minus-strand RNA synthesis. How the triple gene-block proteins interact for virus movement is still elusive. As the potato virus X (PVX) TGBp1 protein gates plasmodesmata, regulates virus translation and is a suppressor of RNA silencing, further research is needed to determine how these properties contribute to propelling virus through the plasmodesmata. Specifically, TGBp1 suppressor activity is required for virus movement, but how the silencing machinery relates to plasmodesmata is not known. The TGBp2 and TGBp3 proteins are endoplasmic reticulum (ER)-associated proteins required for virus movement. TGBp2 associates with ER-derived vesicles that traffic along the actin network. Future research will determine whether the virus-induced vesicles are cytopathic structures regulating events along the ER or are vehicles carrying virus to the plasmodesmata for transfer into neighbouring cells. Efforts to assemble virions *in vitro* identified a single-tailed particle (STP) comprising RNA, coat protein (CP) and TGBp1. It has been proposed that TGBp1 aids in transport of virions or STP between cells and ensures translation of RNA in the receiving cells. PVX is also a tool for studying *Avr-R* gene interactions and gene silencing in plants. The PVX CP is the elicitor for the *Rx* gene. Recent reports of the PVX CP reveal how CP interacts with the *Rx* gene product.

Introduction

The genus *Potexvirus* is one of eight genera belonging to the family *Flexiviridae* (Adams *et al.*, 2004). Members of this family are characterized by flexuous, filamentous virions between 470 and 580 nm in length, built of subunits of a single coat protein (CP). Potexviruses have monopartite, positive-strand RNA genomes encoding five open reading frames (ORFs) (Fig. 1). The 5' end has a methylguanosine cap and the 3' end has a poly(A) tail (Huang *et al.*, 2004; Huisman *et al.*, 1988). The first ORF encodes the viral replicase. The central region of the genome encodes three overlapping ORFs, known as the triple-gene block (TGB). These proteins are required for virus cell-to-cell movement (Verchot-Lubicz, 2005). The final ORF is the viral CP, which is required for virion assembly and virus cell-to-cell movement (Huisman *et al.*, 1988; Santa Cruz *et al.*, 1998).

The ICTV database lists 25 known and 21 tentative member species of the genus *Potexvirus* (Adams *et al.*, 2005). The type member of the genus is *Potato virus X* (PVX). PVX, white clover mosaic virus, foxtail mosaic virus (FMV), cactus virus X (CVX), clover yellow mosaic virus and bamboo mosaic virus (BaMV) have been important for defining the molecular mechanisms controlling virus replication and systemic transport. PVX in particular is widely used as a vector for expression of foreign genes in plants (Baulcombe *et al.*, 1995; Chapman *et al.*, 1992b) and was central for discovering gene silencing and small RNAs in plants (Hamilton & Baulcombe, 1999; Hamilton *et al.*, 2002) and for studying *R* gene-mediated resistance (Bendahmane *et al.*, 1999; Santa Cruz & Baulcombe, 1993). Thus, potexviruses have contributed to our understanding of plant virus life cycles, transport mechanisms in plants, host pathogen resistance and gene silencing. This review highlights the many major research advances surrounding potexviruses and identifies future opportunities for research.

RNA elements regulating potexvirus replication

As another review addresses potexvirus replication (Batten *et al.*, 2003), to avoid redundancy, we focus here on more recent advances in potexvirus replication. The potexvirus replicase is a single protein that contains methyltransferase, RNA helicase and RNA polymerase activities. Isolation of a template-dependent PVX replicase from infected tobacco plants revealed that the PVX replicase associates with cellular membranes (Doronin & Hemenway, 1996; Plante *et al.*, 2000). However, we do not yet know which membrane compartment serves as a centre for virus replication. The machinery controlling membrane association of the potexvirus replicase is also unknown. It is possible that the replicase contains a sequence that controls membrane anchoring of the replication complex or that host factors interacting with the replication complex control membrane binding. Potexviruses encode two proteins, named TGBp2 and TGBp3, that are endoplasmic reticulum (ER)-associated proteins (Ju *et al.*, 2005; Krishnamurthy *et al.*, 2003; Mitra *et al.*, 2003). These proteins are essential for virus movement and have not yet been shown to contribute to replication. However, a recent report showing that TGBp2 of the hordeivirus barley stripe mosaic virus (BSMV) is recruited to the chloroplasts in the presence of

replicase-encoding BSMV RNAs α and γ (Torrance *et al.*, 2006) included speculations that the TGBp2 protein may play a role in replication that has not yet been discovered. Thus, future research may need to explore the relationship of the viral movement proteins with the viral replicase.

Two RNA stem-loop structures, named 5'SL1 and 5'SL2, reside within the first 182 nt and are essential for PVX replication (Kim & Hemenway, 1996; Miller *et al.*, 1998, 1999) (Fig. 1). The PVX 5' non-translated region (NTR) is 84 nt in length (the length varies slightly among potexviruses); the stem-loop structures form from sequences extending into the replicase ORF. The 5'SL1 element is multifunctional, contributing to virus replication, cell-to-cell movement and virion assembly. SELEX (systemic evolution of ligands by exponential enrichment) was used to demonstrate that an intact 5'SL1 and the GAAA sequence within the terminal tetraloop are essential for plus-strand RNA synthesis (Kwon & Kim, 2006; Miller *et al.*, 1999). Changes in the 5'SL1 also affect subgenomic RNA accumulation required for CP production. Evidence of 5'SL1-binding host proteins in tobacco protoplast extracts led to speculations that host proteins may recognize 5'SL1 after virus uncoating and may promote translation or replication of genomic RNA (Kwon & Kim, 2006). As subgenomic RNAs are synthesized, the CP may bind to the 5'SL1, displacing the host factor. As CP is required for virus cell-to-cell movement, binding to this region is important for sequestering RNAs from the viral replicase for transfer into adjacent cells (Kwon & Kim, 2006).

This model is supported by recent evidence that the 5' NTR plays a role in virus cell-to-cell movement (Lough *et al.*, 2006). The 5' segment regulating virus movement was termed an 'RNA zip code' that determines the destination of the cognate RNA within the cell (Lough *et al.*, 2006). The PVX RNA zip code was defined in experiments where plasmids expressing mutant PVX genomes lacking the entire TGB and CP, but containing green fluorescent protein (GFP), were cobombarded with plasmids expressing the entire PVX genome. GFP fluorescence spread between neighbouring cells co-expressing the mutant and wild-type PVX genomes (Lough *et al.*, 2006). A series of deletion mutations was used to identify the RNA segment responsible for virus cell-to-cell spread. Deletions within the first 107 nt (overlapping 5'SL1) of the PVX genome eliminated virus movement, indicating that the 5' NTR is an element in virus movement, as well as replication (Lough *et al.*, 2006).

In addition, 5'SL1 may regulate CP production by base pairing directly with elements in the subgenomic RNA promoters (Fig. 2a, c) (Kim & Hemenway, 1999). Potexviruses have three subgenomic RNAs for expression of TGB and CP (Huisman *et al.*, 1988; Lee *et al.*, 2000). An octanucleotide sequence (AACUAAAC) in the PVX 5' NTR was identified that can base pair with sequences (GUUAAGUU) in the sgRNA1 and sgRNA3 promoters (Batten *et al.*, 2003). Mutations altering the extent of complementarity between these PVX sequences reduced plus-strand and subgenomic RNA synthesis without altering minus-strand RNA synthesis (Kim & Hemenway, 1999) (Fig. 1c). Beyond the 5'SL1 are five ACCAA motifs repeated throughout the 5' NTR and overlapping 5'SL1 (Kim & Hemenway, 1996) (Fig. 1). For PVX, these repeats bind a 54 kDa cellular protein (p54) that is also important for virus replication (Kim *et al.*, 2002).

The PVX 3' NTR is 74 nt in length and contains *cis* elements that control minus-strand as well as plus-strand RNA accumulation. Computer predictions and solution-structure studies identified three stem-loop structures, 3'SL1, 3'SL2 and 3'SL3, in the 3' NTR (Pillai-Nair *et al.*, 2003) (Fig. 1). The 3' elements required for minus-strand synthesis include the entire 3'SL3 stem-loop structure, a hexanucleotide element in the terminal loop of 3'SL3, a U-rich sequence located in the terminal loop and 3' side of the stem in 3'SL2, and the poly(A) tail (Batten *et al.*, 2003; Cheng & Tsai, 1999; Cheng *et al.*, 2002; Chiu *et al.*, 2002; Lin *et al.*, 2005; Pillai-Nair *et al.*, 2003; Sriskanda *et al.*, 1996; White *et al.*, 1992) (Fig. 1). 3'SL1 is not essential for minus-strand RNA synthesis (Pillai-Nair *et al.*, 2003). The U-rich sequence overlapping 3'SL2 was shown to bind host factors that may be required for virus multiplication (Sriskanda *et al.*, 1996). Two cellular factors, chloroplast phosphoglycerate kinase (CPK; also identified as p43) and p51, were found to bind to the 3' NTR of BaMV (Lin *et al.*, 2007). BaMV RNA accumulation was reduced in CPK-silenced plants (Lin *et al.*, 2007).

Two AU-rich sequences neighbouring the poly(A) tail, known as near upstream elements 1 and 2 (NUE1 and NUE2) (Pillai-Nair *et al.*, 2003) (Fig. 1), contribute to plus-strand RNA accumulation (Huang *et al.*, 2001). Mutations within these NUE elements have a greater effect on plus-strand than minus-strand RNA synthesis (Chen *et al.*, 2005). Recent data show that interactions between the hexanucleotide motif in the terminal loop of 3'SL3 and the internal conserved octanucleotide motifs that reside upstream within subgenomic promoter regions are required for minus-strand synthesis (Hu *et al.*, 2007). Similar interactions were reported to occur between a conserved octanucleotide sequence near the 5' terminus and the same subset of internal octanucleotide elements, which are required for plus-strand RNA synthesis (Hu *et al.*, 2007). Thus, conserved elements in both termini interact with the same subset of internal elements for all RNA synthesis (Fig. 2a, b). These observations, along with evidence that elements in the 5' NTR base pair with elements in the subgenomic RNAs, indicate that interactions between distant *cis*-acting elements regulate virus replication and gene expression.

BaMV is one of the few potexviruses known to associate with satellite RNAs (satRNAs) (Lin & Hsu, 1994). BaMV and satBaMV provide novel opportunities for studying long-distance interactions, occurring *in cis* or *in trans*, between RNA elements regulating potexvirus replication and gene expression. satBaMV is a linear RNA of 836 nt and contains a single ORF encoding a 20 kDa protein (Liu *et al.*, 1997; Tsai *et al.*, 1999). Phylogenetic analysis of 60 satBaMV isolates indicated that the satRNAs may be classified into two groups, A and B. The satBaMV 5' NTR contains a hypervariable region that forms a large stem-loop structure and a small stem-loop structure (Annamalai *et al.*, 2003; Yeh *et al.*, 2004). Most isolates have similar stem-loop structures in the 5' NTR (Yeh *et al.*, 2004). Mutational analysis indicated that conserved structures in the 5' NTR of satBaMV are necessary for replication (Annamalai *et al.*, 2003; Yeh *et al.*, 2004). The 5' NTR of satBaMV isolate BSL6, which is the type member for group B, is responsible for interference with BaMV replication (Hsu *et al.*, 2006). BaMV replication was also reduced when the BSL6 5' NTR was inserted into the BaMV infectious clone, demonstrating that the BSL6 5' NTR can function *in cis* to downregulate BaMV replication (Hsu *et al.*, 2006).

Evidence that the BSL6 5' NTR can regulate BaMV replication indicates the possibility that the satBaMV RNA interacts *in trans* with BaMV RNA sequences.

Potexvirus CP: virions, virus movement and single-tailed particles (STPs)

Potexvirus virions are 470–580 nm long, flexuous rods. *In vitro* assembly assays, fibre-diffraction studies, Fourier-transform infrared spectroscopy, Raman spectroscopy and vibrational circular dichroism were used to characterize PVX and narcissus mosaic virus (NMV) virions (Blanch *et al.*, 2002; Kendall *et al.*, 2007; Parker *et al.*, 2002; Shanmugam *et al.*, 2005). Both of these potexviruses have similar structures with high α -helical content. Potexvirus virions have a deeply grooved surface and are bound with water molecules to help maintain surface structure (Baratova *et al.*, 2004). The PVX helix has 8.75 CP subunits per turn of the helix. The NMV viral helix has approximately eight subunits per turn and an outer radius of 55 Å (5.5 nm) (Blanch *et al.*, 2002; Kendall *et al.*, 2007; Parker *et al.*, 2002; Shanmugam *et al.*, 2005).

Papaya mosaic virus (PapMV) and PVX CP subunits expressed in *Escherichia coli* form discs and virus-like empty particles that have been valuable for studying the requirements for virion assembly (Tremblay *et al.*, 2006). Individual CP subunits self-assemble first into helical discs and then into virus-like particles (Lecours *et al.*, 2006; Tremblay *et al.*, 2006). Packaging occurs when these discs bind RNA and then assemble along the central RNA into full-length virions (Tremblay *et al.*, 2006). Virion assembly is triggered when cells contain a sufficient supply of discs and viral RNA in a cell.

The RNA-binding domain of PapMV CP is located between aa 90 and 130 (Lecours *et al.*, 2006). The N terminus is predicted to be exposed at the virion surface and may be crucial for intersubunit interactions and virion assembly. Mild trypsin treatment can easily remove the N-terminal extension (Baratova *et al.*, 2004; Tremblay *et al.*, 2006). There are six serine residues in the N terminus that could be phosphorylation targets for host kinases (Lecours *et al.*, 2006). Phosphorylation of the PVX CP was shown to enhance RNA translation, suggesting that phosphorylation may destabilize subunit interactions, promoting disassembly of the virus (Lecours *et al.*, 2006). There is also evidence that the N terminus is glycosylated in infected plants (Tozzini *et al.*, 1994). Glycosylation is critical for maintaining a surface layer of water molecules (Baratova *et al.*, 2004), which may be important for maintaining virion structure. Changes in CP glycosylation or phosphorylation may alter the hydration shell and, subsequently, virion morphology (Baratova *et al.*, 2004). Such changes may be essential for virion disassembly and may explain abnormal virion structures reported in some electron microscopy studies (Baratova *et al.*, 2004; Chapman *et al.*, 1992a).

The 5' end of the potexvirus genome contains sequences responsible for particle assembly. Initial experiments identified the origin of assembly sequence (OAS) between nt 38 and 47 at the 5' terminus as being sufficient to initiate assembly of PapMV (Sit *et al.*, 1994). Interestingly, this segment lies within the first 107 nt, which also includes the RNA zip code required for virus cell-to-cell movement (Lough *et al.*, 2006), and overlaps the 5' SL1 stem-loop required for virus

replication. However, SELEX was used recently to produce mutant PVX RNAs and to screen for elements that bind CP and assemble virion particles *in vitro*. This study concluded that the 5'SL1 stem-loop acts as the OAS (Kwon *et al.*, 2005). Moreover, it was reported that full-length virions were produced *in vitro* when viral RNA was used, but smaller virus-like particles were detected when full-length, *in vitro*-synthesized transcripts were used (Kwon *et al.*, 2005). These observations suggest that there are additional unknown factors that may limit recovery of full-length particles (Kwon *et al.*, 2005).

In recent years, there have been reports indicating that some plant viruses require more than one RNA element to initiate RNA packaging. Brome mosaic virus (BMV; a bromovirus) requires two RNA elements for virion assembly: the 3' tRNA-like structure and a *cis*-acting element inside the movement protein gene (Choi *et al.*, 2002). Red clover necrotic mosaic virus (RCNMV; a dianthovirus) virions sometimes contain a dimer of genomic RNAs (Basnayake *et al.*, 2006). For tobacco mosaic virus (TMV; a tobamovirus), there is a single OAS near the 3' end of the genome within the movement protein gene. Other tobamoviruses have an OAS within the coat protein gene (Meshi *et al.*, 1981; Srinivasan *et al.*, 2002). Packaging of the potexvirus NMV and BaMV subgenomic RNAs has also been reported (Annamalai & Rao, 2006; Choi & Rao, 2000; Lee *et al.*, 1998; Short & Davies, 1983). Whilst BMV, RCNMV and TMV provide examples of viruses that have different packaging requirements, evidence that BaMV subgenomic RNAs may be packaged into virions reveals that the requirements for potexvirus particle assembly involve more than a single OAS.

An interesting set of experiments have been conducted to explore the effects of TGBp1 on PVX assembly and disassembly. As mentioned briefly above, when PVX virions are added to wheatgerm extracts, the RNA is non-translatable. However, when increasing concentrations of TGBp1 were added, virions were converted into a translatable form (Atabekov *et al.*, 2000; Kiselyova *et al.*, 2003; Rodionova *et al.*, 2003). Thus, a model was proposed suggesting that TGBp1 remodels virus particles, allowing the RNA to be exposed for translation. According to this model, TGBp1 binds to one end of the virion and destabilizes the particle (Rodionova *et al.*, 2003). The presence of TGBp1 at one end of the virion was confirmed by atomic force and electron microscopy. Particles containing TGBp1 at one end of the virion are known as single-tailed particles (STPs) (Karpova *et al.*, 2006). These STPs form *in vitro* when TGBp1 binds to one end of a virion particle, or can assemble *de novo* from a mixture of RNA, CP and TGBp1. This led to a two-step model suggesting that virion assembly precedes polar addition of TGBp1.

As PVX RNA within the STP is translatable (Karpova *et al.*, 2006) and PVX virions are non-translatable, it is possible that TGBp1 attaches to virions early in infection and destabilizes them to promote translation. Further *in vitro* translation experiments showed that translation of virion-derived RNAs can also be triggered by CP phosphorylation (Atabekov *et al.*, 2001). Comparing these two studies, it is easy to imagine that, when virus first enters a cell, CP phosphorylation may trigger translation, thereby initiating the virus infection cycle. Then, as the virus spreads from cell to cell, TGBp1 may function to promote movement of STPs into neighbouring cells or it may function in the receiving cells to ensure that CP-RNA complexes remain translatable (Atabekov *et al.*, 2000, 2001; Rodionova *et al.*, 2003). Thus, when the virus is first introduced

into the plant, virion disassembly may initiate with the aid of host protein kinases, but as the virus continues to replicate and spread, TGBp1 may provide a similar role, eliminating viral dependence on host kinases to initiate viral RNA translation.

Potexvirus CP and virus cell-to-cell movement

Potexviruses use three movement proteins, named TGBp1, TGBp2 and TGBp3, and the viral CP for virus cell-to-cell movement. The TGB proteins have distinct activities, and current research efforts are trying to explain how these are coordinated to move virus from cell to cell and through the vasculature.

The TGB is conserved among viruses belonging to the genera *Potexvirus*, *Hordeivirus*, *Foveavirus*, *Pecluvirus*, *Pomovirus*, *Carlavirus* and *Allexivirus*. These viruses are often described as potexvirus-like or hordeivirus-like in their mechanisms for cell-to-cell movement (Morozov & Solovyev, 2003; Verchot-Lubicz, 2005). Potex-like viruses require CP for cell-to-cell movement and encode a TGBp1 that functions as an RNA-silencing suppressor and a TGBp3 that has a single transmembrane domain. Hordei-like viruses do not require the CP for movement and encode a separate protein that acts as an RNA-silencing suppressor and a TGBp3 that has two transmembrane domains (Morozov & Solovyev, 2003; Verchot-Lubicz, 2005).

It is well documented that plant virus movement proteins dilate plasmodesmata to allow virus cell-to-cell movement (Robards & Lucas, 1990; Roberts *et al.*, 2001). Viral movement proteins interact with a trigger mechanism that expands the pore to allow selective trafficking of large molecules. The most recent models of potexvirus movement show that TGBp1 and CP form a complex with viral RNA, which traffics to the plasmodesmata. The viral ribonucleoprotein (vRNP) complex interacts with cellular proteins at the mouth of the plasmodesmata, which triggers expansion of the pore to allow trafficking between cells (Lucas, 2006; Morozov & Solovyev, 2003; Verchot-Lubicz, 2005). Earlier studies of PVX, CVX and FMV showed that the potexvirus CP resides in plasmodesmata during virus infection (Oparka *et al.*, 1999; Rouleau *et al.*, 1995). Microinjection experiments showed that TGBp1, but not CP, can trigger plasmodesmal gating, suggesting that these proteins act together within the plasmodesmata to promote plasmodesmal gating and virus cell-to-cell transport (Lough *et al.*, 1998, 2000). This led to a model suggesting that a TGBp1–vRNA–CP complex can traffic between cells through plasmodesmata (Lough *et al.*, 1998, 2000). By using electron microscopy, fibrillar structures that were immunoreactive with PVX CP antisera were seen in plasmodesmata (Santa Cruz *et al.*, 1998). Whether the fibrillar material represents virion particles or TGBp1–vRNA–CP complexes or is a cytopathic structure unrelated to the movement complex has not been determined.

The nature of the vRNP complex that traffics through plasmodesmata is still a topic of research. The current model of a TGBp1–vRNA–CP complex was influenced mainly by models of TMV and cauliflower mosaic virus (CaMV; a caulimovirus) in which the respective viral movement proteins bind single-strand RNA non-specifically, forming a linear vRNP structure (Citovsky *et*

et al., 1990, 1991). The viral TMV P30 or CaMV gene I movement proteins bind viral RNA cooperatively, creating an unfolded vRNP structure that may be more compact than a RNP structure built around folded RNA. This extended vRNP structure may be a preferred form to move through plasmodesmata (Citovsky *et al.*, 1992; Citovsky & Zambryski, 1991, 1993). Thus, most depictions of virus movement include a linear vRNP complex (Fig. 3a) that traffics between cells. Initial models of potex- and hordeiviruses suggested that TGBp1 may function similarly to the TMV P30 protein, i.e. as a chaperone carrying viral RNA toward and through the plasmodesmata. As the potexvirus TGBp1 protein has RNA-binding and RNA helicase activities (Kalinina *et al.*, 2002; Leshchiner *et al.*, 2006; Morozov *et al.*, 1999; Rouleau *et al.*, 1994), it was thought initially that TGBp1 may unwind RNA secondary structure while forming the vRNP complex. This model was later changed to include the potexvirus CP as a component of the vRNP complex (Lough *et al.*, 2000).

The potexvirus TGBp1 was shown to promote disassembly of virions and translation of virion-derived RNAs in wheatgerm extracts (Atabekov *et al.*, 2000; Rodionova *et al.*, 2003). This may be considered evidence that an intermediate TGBp1–vRNA–CP complex can exist during infection and may be the complex that moves through plasmodesmata. Alternatively, these data show that TGBp1 helicase activity may be necessary for forming the vRNP complex that traffics between cells, as well as promoting virus translation.

Considering the recent physical characterization of potexvirus virions, STPs, destabilized virus-like particles and the effects of N-terminal modifications on virion structure, it is worth considering that the nature of the vRNP complex may not be resolved completely. Evidence that virus-like particles or modified virions exist *in vivo* as the result of carbohydrate modification, phosphorylation or TGBp1 association (Atabekov *et al.*, 2000, 2001; Baratova *et al.*, 2004; Chapman *et al.*, 1992a; Lecours *et al.*, 2006; Rodionova *et al.*, 2003; Tozzini *et al.*, 1994) makes it reasonable to speculate that the fibrillar structures associating with plasmodesmata identified in earlier studies (Santa Cruz *et al.*, 1998) may represent one of these altered virion forms. It is also worth considering that virus-like particles or modified virions may represent the vRNP complex that traffics from the site of replication toward and across the plasmodesmata.

Potexvirus TGBp1, TGBp2 and TGBp3 provide separate activities aiding virus cell-to-cell movement

TGBp1 is a multifunctional protein that is required for virus cell-to-cell movement. We already mentioned that TGBp1 triggers expansion of plasmodesmata (allowing transfer of virus and other molecules between cells), has RNA helicase activity and may be a component of a vRNP complex transferring infectious units between cells (Angell *et al.*, 1996; Howard *et al.*, 2004; Lough *et al.*, 1998; Yang *et al.*, 2000). TGBp1 is also a suppressor of RNA silencing (Voinnet *et al.*, 2000). There is evidence to suggest that TGBp1 interacts with RDR6, which is a factor in short interfering RNA (siRNA) production and meristem exclusion of some viruses (including PVX) (Qu *et al.*, 2005; Schwach *et al.*, 2005; Xie & Guo, 2006). RDR6, DCL4 and HEN1 are factors required for initiation or maintenance of virus-induced gene silencing in growing tissues.

DCL4 produces 21 nt viral siRNAs that are amplified by RDR6 (Blevins *et al.*, 2006; Dunoyer *et al.*, 2005). The amplified 21 nt siRNAs then target viral RNA for silencing and spread to the shoot apical meristem, where they act to restrict virus infection. Mutations impeding TGBp1 suppressor activity also inhibited virus movement, indicating that these processes are coordinated (Bayne *et al.*, 2005). Other viral suppressors, such as potyvirus HC-Pro, tombusvirus P19 and closterovirus p21, bind siRNAs and prevent microRNA methylation by HEN1 (Blevins *et al.*, 2006; Lakatos *et al.*, 2004, 2006; Merai *et al.*, 2006; Yu *et al.*, 2006). CaMV has a silencing suppressor that stabilizes double-stranded RNA (dsRNA) products of RDR6 and impairs processing of long dsRNAs by DCL4 (Blevins *et al.*, 2006). Whether the potyvirus TGBp1 blocks amplification of the 21 nt siRNAs or acts downstream of RDR6 is still uncertain, but it is likely that its role as a silencing suppressor protects the replicating virus from being a target for RNA silencing. This may be sufficient to promote cell-to-cell spread of virus infection, or there may be additional machinery that has yet to be discovered, which further links the viral plasmodesmal transport machinery with RNA silencing.

The potyvirus TGBp2 and TGBp3 proteins are ER-binding proteins. Amino acid sequence analyses determined that TGBp2 has two transmembrane domains and that TGBp3 has a single, N-terminal transmembrane domain (Krishnamurthy *et al.*, 2003; Mitra *et al.*, 2003). Mutations disrupting membrane association of these proteins also inhibit virus movement, indicating that ER association is important (Krishnamurthy *et al.*, 2003; Mitra *et al.*, 2003).

GFP was fused to TGBp2 and introduced into the PVX genome and into pRTL2 plasmids. In protoplasts and plants inoculated with PVX-GFP : TGBp2 or transfected with pRTL2-GFP : TGBp2, fluorescence was mainly in small, granular-type vesicles and the ER (Ju *et al.*, 2005). These granular vesicles aligned on actin filaments, suggesting that they may traffic along the cytoskeleton toward the plasmodesmata. Electron microscopy confirmed that these are ER-derived vesicles induced by GFP-TGBp2. These vesicles appeared to contain ribosomes, were immunoreactive with GFP and BiP (an ER-resident chaperone) antisera (Ju *et al.*, 2005) and were unaffected by brefeldin A, which is known to dissolve Golgi (Mitra *et al.*, 2003). Deletion of conserved amino acids in the central region of the TGBp2 protein (located between the two transmembrane domains) blocked GFP-TGBp2 accumulation of fluorescence in the small, granular-type vesicles and inhibited virus cell-to-cell movement. These data indicate that the granular-type vesicles play an essential role in virus movement (Ju *et al.*, 2007). Substitution mutations individually replacing conserved residues Tyr55, Asp57, Thr59, Lys60, Ile62 or Tyr64 were each sufficient to eliminate TGBp2 association with granular vesicles and inhibit virus cell-to-cell movement (Ju *et al.*, 2007). These data provide the first evidence indicating that granular vesicles induced by the PVX TGBp2 protein are necessary for virus movement (Ju *et al.*, 2007).

In tobacco leaves expressing GFP-TGBp3 fusions, fluorescence was mainly in the ER network (Ju *et al.*, 2005; Krishnamurthy *et al.*, 2003). However, when plasmids encoding GFP-TGBp3 were co-expressed with PVX, fluorescence was seen in granular vesicles similar to those induced by TGBp2 (Schepetilnikov *et al.*, 2005). One explanation is that TGBp2 may direct TGBp3 into the same ER-derived vesicles during virus infection. Whilst studies have shown that TGBp2 and TGBp3 sometimes colocalize (Schepetilnikov *et al.*, 2005; Zamyatnin *et al.*, 2002),

no evidence has yet been presented to indicate whether the TGBp2-related vesicles contain TGBp1, vRNA, CP or virus-like particles.

The potato mop top virus (PMTV) TGBp2 and TGBp3 proteins were reported to associate with motile granules, which traffic toward the plasmodesmata, early in virus infection (Haupt *et al.*, 2005). Later, the PMTV TGBp2 and TGBp3 proteins were seen associating with endocytic vesicles budding from the cell wall (Haupt *et al.*, 2005). A model for PMTV was proposed, indicating that the TGBp2 and TGBp3 proteins may be retrieved from the plasma membrane by the endosome and then returned to the site of virus replication for repeated rounds of vRNP trafficking (Haupt *et al.*, 2005). As confocal images show similar granular bodies in PMTV- and PVX-infected cells (Haupt *et al.*, 2005; Ju *et al.*, 2005), it is worth considering that the granular bodies depicted in the PMTV model carrying infectious agents to the plasmodesmata may be the same TGBp2-induced vesicles as were described for PVX (Fig. 3a). There is no evidence yet that potexviruses use the endosome to retrieve and recycle movement proteins.

One important consideration of this model is the lack of knowledge concerning the location where these potex-like and hordei-like viruses replicate in the cell. As endocytic vesicles often fuse with the Golgi, ER or vacuolar membranes, it would be easy to imagine that the TGB proteins are recycled to these locations to acquire more vRNP cargo, if virus replication occurs along these membranes. However, if these viruses replicate along membranes of the mitochondria, chloroplast, peroxisome or other organelles, then recycling of viral factors would require the endocytic vesicles to be redirected to new locations for recycling of the viral factors. Thus, our understanding of viral protein recycling may be reshaped by future investigations to identify the locations used by potex- and hordei-like viruses for replication.

Another important consideration of this model is the lack of direct evidence that the potexvirus TGB and CP interact directly with each other. There is little reported evidence indicating that all four proteins and vRNA form a 'transport' complex. As we learn more about the separate functions of these proteins, more questions are raised about how these activities are coordinated to promote cell-to-cell trafficking of viral RNA. Do TGBp1, TGBp2 and TGBp3 form a membrane-bound complex that traffics laterally along the ER toward and through the plasmodesmata, as some researchers have suggested? Alternatively, the potexvirus movement proteins and CP may coordinate their activities over space and time, rather than forming a single complex directly (Verchot-Lubicz, 2005). Evidence that CP accumulates inside plasmodesmata and that TGBp1 gates plasmodesmata suggests that these two proteins may act independently of TGBp2 and TGBp3 to regulate changes in the plasmodesmal aperture (Verchot-Lubicz, 2005). Virions or viral RNA may be transported later within the cell toward the plasmodesmata. Whilst it is reasonable to consider that TGBp2 and TGBp3 coordinate to transport a TGBp1–CP–vRNA complex laterally along the ER toward the plasmodesmata (Lucas, 2006; Morozov & Solovyev, 2003), recent evidence that PVX TGBp2-induced vesicles are necessary for virus cell-to-cell movement (Ju *et al.*, 2007) raises obvious questions about the nature of their cargo and whether these vesicles traffic infectious agents between cells. The TGBp2 protein of the pomovirus PMTV binds single-stranded RNA *in vitro*, colocalizes with TGBp3 and does not interact with TGBp1 (Cowan *et al.*, 2002). Whilst there is no clear

evidence that PMTV TGBp2 induces vesicles similar to the PVX TGBp2-related structures, if PVX TGBp2 has an ability to bind RNA similar to that of the PMTV TGBp2, then it becomes reasonable to consider that the TGBp2-induced vesicles might traffic viral RNA toward the plasmodesmata. A model in which vesicles trafficking viral RNA to the plasmodesmata seems to oppose the model of a TGBp1–CP–vRNA complex trafficking along the ER to the plasmodesmata. The vesicle-transport model suggests that TGBp1 is not necessary for trafficking viral RNA within the cell, although it may play a role in guiding RNA across the plasmodesmata once it has exited the vesicles. Thus, it is reasonable to consider that the TGBp2-induced vesicles play an alternative role in promoting virus cell-to-cell movement. Perhaps TGBp2-related vesicles act upstream of the movement process to regulate virus replication. It is also possible that the TGBp2-induced vesicles play a role in modulating the ER stress responses (Ju *et al.*, 2005) or other events in the virus life cycle, thereby enabling virus cell-to-cell movement.

PVX CP: eliciting resistance

The PVX CP is the elicitor for *Rx*-mediated resistance in potato. The cloning of *Rx1* and *Rx2* was reported by Bendahmane *et al.* (1999, 2000). Since then, studies of PVX CP and *Rx* have been central to defining how elicitor recognition triggers downstream signals leading to disease resistance. *Rx* is a CC–NBS–LRR protein that prevents virus accumulation in inoculated leaves and in protoplasts. *Rx* normally causes extreme resistance, but when the PVX CP is expressed from the CaMV 35S promoter, we see hypersensitive resistance (HR) (Bendahmane *et al.*, 1999, 2000). As PVX is the target of *Rx* resistance, it appears that *Rx* acts rapidly to block virus replication and further CP expression, whilst overproduction of CP from the CaMV 35S promoter leads to HR (Bendahmane *et al.*, 1999, 2000). Mutations within the PVX CP eliminating Lys121 and Arg127 disrupted *Rx*-mediated resistance and reduced virus infection of plants lacking *Rx* (Bendahmane *et al.*, 1995).

Analysis of the separate contributions of the CC, NBS and LRR regions of *Rx* to PVX CP-binding and downstream-signalling events leading to HR provided new insights into how plant viral *R*–*Avr* interactions function. Briefly, the *Rx* protein has a folded resting-state conformation that is altered by interactions with the PVX CP (Moffett *et al.*, 2002; Rairdan & Moffett, 2006; Rathjen & Moffett, 2003). The CP causes the *Rx* protein to become unfolded, exposing the NBS–LRR domains. This change in protein conformation causes a change in the nucleotide-binding status of the protein, whilst activating the signalling cascade (Moffett *et al.*, 2002; Rathjen & Moffett, 2003). Details of this PVX–*Rx* model and other viral *R*-gene interactions have been reviewed elsewhere (Rathjen & Moffett, 2003).

PVX is a tool for studying RNA silencing

PVX has made a huge contribution to the discovery of RNA silencing. A turning point in these studies occurred when the β -glucuronidase (GUS) reporter was introduced into PVX infectious

clones (Baulcombe *et al.*, 1995; Chapman *et al.*, 1992b). When PVX-GUS was inoculated to GUS-transgenic tobacco, GUS expression was eliminated (Baulcombe, 1996; English *et al.*, 1996). Recombinant PVX viruses containing either phytoene desaturase (PDS) or GFP genes provided the first evidence that viruses can induce silencing of the GFP transgene or the endogenous PDS gene in plants (Ruiz *et al.*, 1998; Voinnet *et al.*, 1998). Studies such as this one led to the development of VIGS (virus-induced gene silencing) technology. VIGS uses viral vectors, such as PVX, carrying inserts derived from host genes to shut off expression of the cognate host gene (Angell & Baulcombe, 1997). Plants are inoculated with the recombinant PVX, triggering RNA silencing that results in degradation of the target host mRNA. This effectively downregulates host-gene expression without altering the host DNA. PVX has been widely used as a VIGS vector to downregulate expression of target genes for studying their contributions to specific host traits.

PVX amplicons were developed as a tool for downregulating host-gene expression in entire plants. Essentially, amplicons are transgenically expressed PVX genomes with the gene of interest inserted into the genome. Recombinant PVX viruses expressed from transgenes were shown to silence host genes uniformly throughout the entire plant (Angell & Baulcombe, 1997, 1999). Adding viral suppressor proteins through *Agrobacterium* infiltration or by crossing with other transgenic plants can restore protein expression. Combining the use of amplicons with *Agrobacterium* delivery of silencing suppressors creates a system that provides researchers with the capacity to switch target genes off and on for analysis of gene function (Mallory *et al.*, 2002).

PVX played a key role in identifying small RNAs in plants. In the first study demonstrating the existence of small RNAs in plants, PVX-specific 25 nt antisense RNAs were found in inoculated tobacco leaves (Hamilton & Baulcombe, 1999; Hamilton *et al.*, 2002). Later, two populations of 21 and 25 nt siRNAs were found in PVX-infected plants. Accumulation of the 25 nt siRNA was controlled by the PVX TGBp1 silencing suppressor. When TGBp1 was deleted from the PVX genome, the 25 nt siRNA accumulated and systemic silencing occurred (Voinnet *et al.*, 2000). Thus, TGBp1 was determined to be a suppressor of systemic RNA silencing by regulating accumulation of the 25 nt siRNA. The shorter 21 nt siRNA plays a role in local RNA silencing, whilst the 25 nt siRNA is a factor in systemic silencing (Hamilton & Baulcombe, 1999; Hamilton *et al.*, 2002; Voinnet *et al.*, 2000).

Perspectives

Potexviruses are powerful tools in research and will continue to provide new information about virus–host interactions. In recent years, the 5' NTR has been identified as a central factor in virus replication, virion assembly and virus movement (Batten *et al.*, 2003; Hsu *et al.*, 2006; Lough *et al.*, 2006; Yeh *et al.*, 2004). The region surrounding and including 5'SL1 binds host factors required for replication and CP. Evidence that this sequence element is required for virus cell-to-cell movement causes us to wonder whether virion assembly, virus-like particles or

STPs are required for virus movement, whether replication complexes are carried through the plasmodesmata and whether the host factors identified in gel-shift assays may be factors in movement, as well as replication. Further research is needed to discover whether viral and host factors compete for binding to the 5' NTR and how the 5' NTR contributes to regulating the timing of events involved in replication and movement.

Further research is also needed to understand the role of the endomembrane network in virus replication and movement. Whilst there is significant evidence using potex-, hordei-, pomo- and tobamoviruses showing that the ER is necessary for virus movement, we do not know whether viruses move laterally along the ER across the plasmodesmata or whether viruses move from the ER into vesicles that traffic to the plasmodesmata (Liu *et al.*, 2005; Verchot-Lubicz, 2005). The best evidence to support a role for vesicles in trafficking is the mutations in PVX TGBp2 that eliminate production of granular vesicles and virus movement. The role and identity of vesicles in potexvirus transport are important questions that need to be addressed. There has been a significant amount of confocal microscopic research, conducted by using potex- and hordei-like viruses, that identified motile granular and endocytic bodies relating to the TGBp2 and TGBp3 proteins. There is only one study using electron microscopy showing that the granules may be vesicles. Further high-resolution work is needed to identify the origin and nature of these granular bodies and to determine whether they are vesicle containers carrying virus to the plasmodesmata. With such little information available concerning the nature and origin of these granular structures, it is also reasonable to consider that these membrane granules are induced by host defences to block the actions of the viral MPs. Thus, we cannot yet be certain that they are containers carrying infectious agents between cells.

Recent studies of the tobamovirus TMV showed that membrane-bound replication complexes are carried along microfilaments toward, and possibly through, the plasmodesmata (Kawakami *et al.*, 2004; Liu *et al.*, 2005). By using confocal microscopy, researchers have describe motile, fluorescent bodies carrying virus to the periphery of the cell (Kawakami *et al.*, 2004; Liu *et al.*, 2005). However, the debate over the nature of these bodies for TMV is similar to the discussion surrounding the granular vesicles seen in potex- and hordei-like virus infections. Questions include whether these fluorescent bodies/granules are vesicles budding from the ER or membrane protrusions that traffic laterally along the ER toward and through the plasmodesmata (Kawakami *et al.*, 2004; Liu *et al.*, 2005). Could the motile fluorescent bodies seen in TMV-infected cells and the fluorescent granules seen in potexvirus-infected cells originate from membranous compartments other than the ER? If virus replication is associated with another membranous compartment, could the TGBp2/TGBp3 proteins cause membrane invaginations that then migrate along the ER to reach the plasmodesmata? Defining the role of the ER in TMV movement is more difficult for TMV than for potexviruses because all TMV movement functions are contained in one protein. As potexviruses use four proteins to promote virus cell-to-cell movement, by studying individual proteins, researchers have a better opportunity to deconstruct the components of the plasmodesmal transport pathway and study each subcellular component in a manner that may not be achievable with viruses encoding a single movement protein.

We know very little about how PVX interacts with host proteins to mediate virus replication or to elicit disease resistance. There is some exciting new research describing how PVX CP interacts with the *Rx* protein to induce extreme resistance. Evidence that disassembly of PVX particles may involve phosphorylation of the CP offers opportunities to study how cellular modifications of the CP affect elicitor recognition by *Rx*. Other CP modifications, such as glycosylation, TGBp1 association and the water shell, affect intersubunit interactions and virion morphology in a manner that may impact *R-Avr* gene interactions. It is easy to imagine that CP modifications may be necessary for *R-Avr* gene interactions or that the virus relies on these modifications to avoid detection. Whilst studies have shown that free CP can elicit *Rx* resistance, we do not know whether intact particles can bind to the host receptor.

Researchers have begun limited experiments using microarray technology to identify host factors in potexvirus infection. A single study was conducted using *Arabidopsis* gene chip microarrays to identify host genes that were induced by several RNA viruses (Whitham *et al.*, 2003). Similar genes were induced by PVX, turnip vein-clearing virus, oilseed rape virus, cucumber mosaic virus and turnip mosaic virus, suggesting that similar factors may contribute to virus replication, movement and host defences. Among the list of induced factors included a putative pectin methylesterase and β -1,3-glucanases (Whitham *et al.*, 2003). This is interesting because a host factor named TIP, which interacts β -1,3-glucanase, was identified in a yeast two-hybrid screen to interact with PVX TGBp2 (Fridborg *et al.*, 2003). As β -1,3-glucanase regulates callose deposition, it is arguable that the PVX TGBp2 protein promotes virus movement by modulating callose deposition (Fridborg *et al.*, 2003). Thus, the limited available information derived from *Arabidopsis* experiments may provide a list of candidate host factors contributing to virus infection. With the recently available potato (*Solanum tuberosum*) cDNA microarray from The Institute of Genomic Research, researchers may begin to identify host factors induced by potexviruses in solanaceous hosts. The potato cDNA microarray was employed successfully to identify genes induced by *Sonchus* yellow net virus and *Impatiens* necrotic spot virus in *Nicotiana benthamiana* (Senthil *et al.*, 2005). Combining data obtained from microarrays with high-throughput gene silencing provides a powerful opportunity for the future to identify host factors contributing to virus replication, cell-to-cell movement and antiviral defences (Senthil *et al.*, 2005).

Finally, PVX will continue to be an important vector for studying host-gene expression and RNA silencing in plants. A far greater number of publications have used PVX as a vector for expressing foreign genes than have been mentioned here. Because of its broad host range, it is reasonable to consider that its use as a vector for research in gene silencing and other fields will continue.

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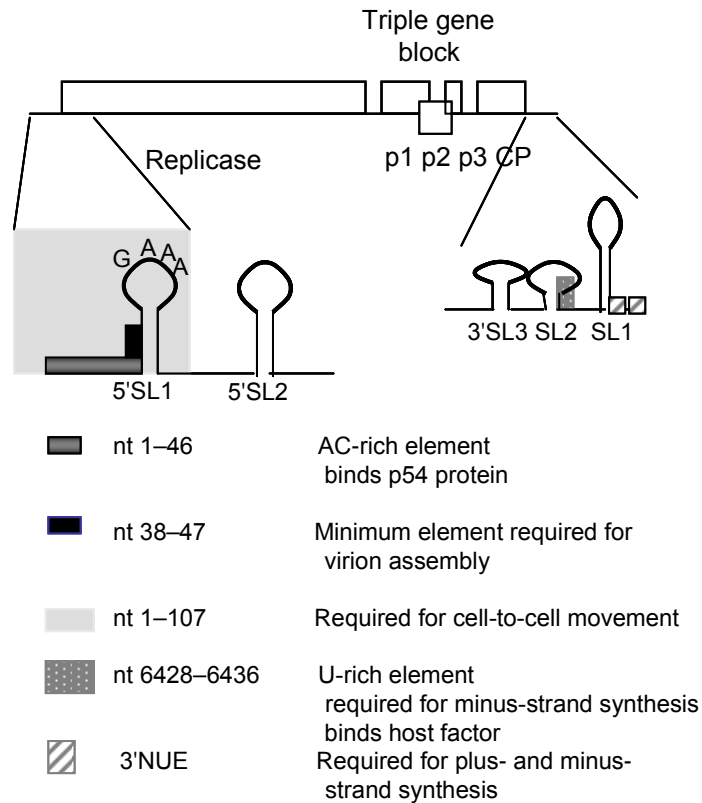


Fig. 1. Diagrammatic representation of the genome of *Potato virus X*, the type member of the genus *Potexvirus*. Lines indicate RNA sequence and boxes represent ORFs. The name of each ORF is listed above the genome. 5' and 3' NTR elements are highlighted (specifically, 5'SL1, 5'SL2, 3'SL1, 3'SL2 and 3'SL3 are indicated). Shaded and cross-hatched boxes represent important elements in the 5' and 3' NTRs controlling RNA synthesis, encapsidation and movement. The lengths of the 5' and 3' elements are described for PVX. The overall length of the 5' and 3' NTR may vary slightly among potexviruses, but the nature of the 5' and 3' elements has not been disputed for the viruses that have been used to characterize these elements. The PVX vector was constructed by duplicating the CP subgenomic promoter and adding a polylinker between the TGBp3 and CP coding sequence (Baulcombe *et al.*, 1995). Foreign genes are inserted into the polylinker.

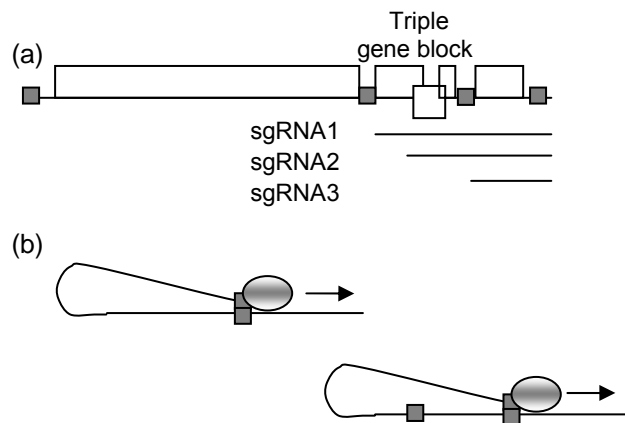


Fig. 2. Diagrammatic representation of long-distance interactions regulating RNA synthesis. (a) Grey boxes indicate elements in the genome that are complementary. The three subgenomic RNAs are shown below the viral genome. (b) Interactions between the 5' NTR and the subgenomic RNA1 and RNA3 promoters. These interactions are necessary for subgenomic RNA synthesis by the replicase (shaded oval).

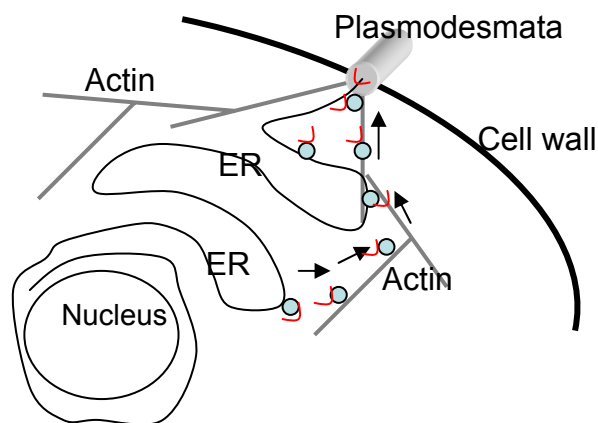


Fig. 3. Model of potexvirus movement. Diagrammatic representation of TGBp2-induced vesicles (blue balls) carrying virus to the plasmodesmata for transport into the neighbouring cell. There is evidence that TGBp3 associates with these vesicles (Schepetilnikov *et al.*, 2005). The red line represents the ribonucleoprotein complex associating with these vesicles according to Haupt *et al.* (2005) and Lough *et al.* (2000). However, recent work by Karpova *et al.* (2006) and Rodionova *et al.* (2003) presents the possibility that STPs associate with the TGBp2/TGBp3-containing vesicles. However, we do not yet know whether other viral proteins or RNA are tethered to these structures. Recent models suggest that these vesicles bud from the ER and move along the actin network to the periphery of the cell.