Unusual Long-Distance Movement Strategies of *Potato mop-top virus* RNAs in *Nicotiana benthamiana*

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The *Potato mop-top virus* (PMTV) genome encodes replicase, movement, and capsid proteins on three different RNA species that are encapsidated within tubular rod-shaped particles. Previously, we showed that the protein produced on translational readthrough (RT) of the coat protein (CP) gene, CP-RT, is associated with one extremity of the virus particles, and that the two RNAs encoding replicase and movement proteins can move long distance in the absence of the third RNA (RNA-CP) that encodes the capsid proteins, CP and CP-RT. Here, we examined the roles of the CP and CP-RT proteins on RNA movement using infectious clones carrying mutations in the CP and CP-RT coding domains. The results showed that, in infections established with mutant RNA-CP expressing CP together with truncated CP-RT, systemic movement of the mutant RNA-CP was inhibited but not the movement of the other two RNAs. Furthermore, RNA-CP long-distance movement was inhibited in a mutant clone expressing only CP in the absence of the CP-RT polypeptide. CP-RT was not necessary for particle assembly because virions were observed in leaf extracts infected with the CP-RT deletion mutants. RNA-CP moved long distance when protein expression was suppressed completely or when CP expression was suppressed so that only CP-RT or truncated CP-RT was expressed. CP-RT but not CP interacted with the movement protein TGB1 in the yeast two-hybrid system. CP-RT and TGB1 were detected by enzyme-linked immunosorbent assay in virus particles and the long-distance movement of RNA-CP was correlated with expression of CP-RT that interacted with TGB1; mutant RNA-CP expressing truncated CP-RT proteins that did not interact with TGB1 formed virions but did not move to upper non-inoculated leaves. The results indicate that PMTV RNA-CP can move long distance in two distinct forms: either as a viral ribonucleoprotein complex or as particles that are most likely associated with CP-RT and TGB1.

Potato mop-top virus (PMTV) is the type species of the genus *Pomovirus*; other member species include *Beet virus Q*

(BVQ), *Beet soil-borne virus* (BSBV), and *Bean broad necrosis virus* (BBNV) (Koenig and Lesemann 2005; Savenkov 2002; Scott et al. 1994). PMTV is the best studied of these in terms of cell-to-cell movement and transmission (Koenig and Lesemann 2005; Torrance 2008). The *Pomovirus* genome comprises three positive-sense, single-stranded RNAs encapsidated in tubular, rod-shaped particles. The PMTV genome contains eight open reading frames (ORF). The longest genomic RNA, RNA 1, encodes viral replicase (Savenkov et al. 1999). A second RNA species encodes the triple gene block (TGB) of movement proteins TGB1, TGB2, and TGB3 and an ORF encoding a 8-kDa cysteine-rich protein (CRP) referred to here as RNA-TGB. A third species of RNA encodes virus coat protein (CP) and a larger polypeptide which is produced by translational read-through (RT) of the CP amber stop codon (CP-RT) (Kashiwazaki et al. 1995) called RNA-CP. The central part of the ORF encoding the CP-RT is prone to spontaneous deletions, particularly during serial manual passage, and, in most natural isolates, the RNA-CP is longer and encodes a larger CP-RT polypeptide (89 kDa) (Sandgren et al. 2001) than the original sequenced laboratory isolate (Kashiwazaki et al. 1995). This observation has caused some confusion in nomenclature of the smaller two RNA species, which are identified here by the proteins they encode for clarity.

PMTV TGB proteins belong to the Hordei-like group of TGB movement proteins and all three are involved in virus cell-to-cell movement (Haupt et al. 2005; Morozov and Solov'yev 2003; Zamyatin et al. 2004). We have demonstrated that the CP and CP-RT are dispensable for replication, accumulation, and cell-to-cell and systemic (long-distance) movement of PMTV RNA 1 and RNA-TGB in plants (McGeachy and Barker 2000; Savenkov et al. 2003). Paradoxically, PMTV is thought to be transmitted in the form of virus particles by the natural vector *Spongospora subterranea* and the CP-RT was shown to be associated with one extremity of the virus particles (Cowan et al. 1997), with a domain in the CP-RT being essential for transmission (Reavy et al. 1998). The aim of this study was to investigate the role of the virus CP and CP-RT in the systemic movement of viral RNAs.

RESULTS

Deletions in the CP-RT domain inhibit the long-distance movement of RNA-CP.

Previously, we have shown that RNA1 and RNA-TGB, when inoculated alone, are capable of moving long distance in *Nicotiana benthamiana* in the absence of CP. To examine the role of CP and CP-RT in PMTV systemic movement, a set of mutant clones was engineered that contained deletions in the

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C-terminal part of the RT domain (Fig. 1). Deletions that varied in size between 330 and 1,386 nucleotides (nt) were made by polymerase chain reaction (PCR)-based mutagenesis of the RT-encoding part of an infectious full-length RNA-CP clone. The resulting six mutants, called del-1 to del-6 (Fig. 1), all terminate at nucleotide position 2,730 conserving the integrity of the 3' untranslated region (UTR). In these and subsequent experiments, RNA-CP transcripts from each of the mutants were prepared and inoculated onto leaves of *N. benthamiana* along with transcripts of RNA 1 and RNA-TGB. Inoculated and upper noninoculated leaves were analyzed by Western and Northern blotting 14 and 28 days postinoculation (dpi).

None of the mutants induced yellow mosaic symptoms typical of PMTV infection and CP antigen was not detected in upper noninoculated leaves by either enzyme-linked immunosorbent assay (ELISA) (data not shown) or Western blotting (Fig. 2A). CP-related polypeptides of the expected size were

Fig. 1. Schematic representation of the *Potato mop-top virus* (PMTV) RNA-CP wild-type (WT) and mutants. The locations of the sequences deleted in PMTV-T isolate are denoted by shading. The position of a 33-amino-acid region within subdomain 3 (SD3) is indicated with an asterisk. The position of the suppressible UAG termination codon is indicated by a short arrow. The deletions in the readthrough (RT) open reading frame are denoted with slanted lines. SD1, SD2, and SD3 = subdomains 1, 2, and 3, respectively.

detected in Western blots of samples prepared from inoculated leaves (Fig. 2A, upper panel, arrow). In addition, wild-type (wt) virus and del-3 mutant produced CP-related polypeptides corresponding to the predicted size of the CP-RT and truncated CP-RT (Fig. 2A, upper panel, lanes 1 and 5). The five other mutants failed to accumulate detectable amounts of the C-ter-

Fig. 2. Effects of unidirectional deletions in viral proteins (coat protein [CP] and readthrough of the CP gene [CP-RT]) on the accumulation and long-distance movement of viral RNAs. **A**, Immunoblot analysis of *Nicotiana benthamiana* plants inoculated with *Potato mop-top virus* (PMTV) mutants and wild-type (wt) virus. Total protein from inoculated (upper panel) and upper noninoculated (systemic) leaves (lower panel) at 14 days postinoculation (dpi) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting with anti-CP immunoglobulin Gs. The positions of CP and CP-RT are shown on the left of the blots. **B**, Northern blot analysis of progeny viral RNAs accumulated in *N. benthamiana* plants inoculated with various mutant RNA-CP transcripts together with wt RNA 1 and RNA-TGB transcripts. Total cellular RNA was extracted from upper noninoculated leaves of *N. benthamiana* plants inoculated with PMTV mutants and wt virus 14 dpi (samples denoted with a) or 28 dpi (samples denoted with b). The Northern blots were analyzed with PMTV 3' untranslated region (UTR)-specific probe, which detects all three genomic RNAs (upper panel), and with RNA-CP-specific probe (lower panel). Asterisks indicate the position of RNA 1 (*) and RNA-TGB (**) (lanes 4 through 12) or RNA-TGB and RNA-CP (lines 2 and 3), which are similar in size and coincide on the Northern blot; # indicates RNA-TGB defective RNA as described (Torrance et al. 1999). Wt-1 and wt-2 are duplicated samples. Reproducible results were obtained in duplicate experiments.

minally truncated CP-RT (Fig. 2A, upper panel, lanes 2–4, 6, and 7). Possibly, these polypeptides were unstable in vivo because they do not retain the normal RT C-terminus and were rapidly degraded in the plant cell. A similar phenomenon was reported for *Beet necrotic yellow vein virus* (BNYVV) CP-RT (Schmitt et al. 1992). Northern blotting revealed accumulation of RNA-CP in inoculated leaves (data not shown) but not in

the systemically infected leaves (Fig. 2B) despite the fact that both RNA1 and RNA-TGB were detected in systemically infected leaves in all the samples analyzed (Fig. 2B, upper panel). Additionally, all the mutants replicated and accumulated in protoplasts of *N. benthamiana* (data not shown).

The only mutant that accumulated detectable amounts of the C-terminally truncated CP-RT in inoculated leaves had a

Fig. 3. Effects of mutations in the *Potato mop-top virus* (PMTV) RNA-CP on viral RNA long-distance movement. **A**, Immunoblot analysis of *Nicotiana benthamiana* plants inoculated with del-SD2&3 and del-SD2 mutants and wild-type (wt) virus. At 14 days postinoculation (dpi), equal amounts of the total protein from inoculated (upper panels) and upper noninoculated leaves (lower panels) was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting with anti-CP immunoglobulins G (IgGs). The positions of CP, readthrough of the CP gene (CP-RT), and truncated CP-RTs are shown on the left of the blots. Del-SD2 is analogous to PMTV-T isolate and lacks subdomain 2 (in-frame deletion), and del-SD2&3 mutant lacks subdomains 2 and 3. **B**, Northern blots showing the accumulation of the PMTV genomic RNAs and RNA-CP mutants in inoculated leaves (upper panel) and upper noninoculated leaves (middle and lower panels) are shown. Total cellular RNA was extracted from inoculated leaves and upper noninoculated leaves. The Northern blots were analyzed with RNA-CP-specific probe (upper and middle panels) and with PMTV 3' untranslated region (UTR)-specific probe, which detects all three genomic RNAs (lower panel). The identities of wt and mutant RNAs are shown to the side of the image. The positions of the RNAs are indicated on the right. **C**, Appearance of PMTV symptoms in *N. benthamiana* infected with CP-RT Only mutant. Severe vein necrosis is evident 14 dpi. **D**, Appearance of PMTV symptoms (yellow mosaic) in *N. benthamiana* infected with wt virus. **E**, Immunoblot analysis of *N. benthamiana* plants inoculated with mutant CP-RT Only and wt virus. At 14 dpi, total protein from inoculated (i) and upper noninoculated leaves (s) was subjected to SDS-PAGE and immunoblotting with anti-CP IgGs. Consistent results were obtained in five experiments.

1,024-nt deletion (Fig. 1, del-3), which overlaps with subdomain 2 (SD2), the region proposed to be a recombination hotspot in PMTV RNA-CP (Sandgren et al. 2001). This data suggested that not all mutations could be tolerated by the virus in terms of CP-RT stability in plants. We have previously shown that the isolate PMTV-T, which had been maintained by manual passage for many years, has a 542-nt in-frame deletion in SD2 compared with the field PMTV-S and PMTV-Sw isolates (Fig. 1) (Reavy et al. 1998; Sandgren et al. 2001), and this deletion spans the recombination hotspot (Sandgren et al. 2001). Thus, based on the fact that CP-RT of the isolate PMTV-T is stable and can be detected in the virus particles (Cowan et al. 1997), we reasoned that an in-frame deletion, encompassing SD2, should render truncated CP-RT stable. New deletion variants were engineered (del-SD2 and del-SD2&3) and are shown diagrammatically in Figure 1. Analysis of plants inoculated with these mutants showed that CP-related polypeptides of the expected size were detected in Western blots of samples prepared from inoculated leaves. Virus proteins (CP and CP-RT) and RNA-CP were readily detected in the inoculated leaves of *N. benthamiana* plants inoculated with del-SD2 and del-SD2&3 mutants (Fig. 3A, upper panel, lanes 3 through 6; B, upper panel, lanes 5 and 6). As expected, del-SD2 mutant accumulated in upper noninoculated leaves (Fig. 3A, lower panel, lanes 5 and 6; B, middle panel, lane 6) and induced mild symptoms with no visible yellow mosaic similar to those obtained with PMTV-T (data not shown).

Neither CP, CP-RT (Fig. 3A, lanes 3 and 4), nor RNA-CP (Fig. 3B, lane 5) were detected in upper noninoculated leaves of plants inoculated with del-SD2&3 mutant, indicating that the region of 1,756 to 2,730 nt was necessary for long-distance movement of RNA-CP. As before, RNA 1 and RNA-TGB were detected in upper leaves regardless of the RNA-CP construct co-inoculated with them (Fig. 3B, lower panel).

RNA-CP that does not express CP or that expresses CP-RT but not CP alone moves to upper leaves.

To further investigate the role of the individual proteins CP and CP-RT on RNA movement, four additional mutants were prepared (Fig. 1): i) No Protein, which carries two stop codons (UAA and UAG) at the beginning of the CP ORF to prevent CP and CP-RT expression; ii) CP Only, which possesses an additional UAA stop juxtaposed with a suppressible UAG codon to prevent readthrough of the CP termination codon and CP-RT production; iii) del-RT, which contains an extensive deletion in the 5'-proximal part of the RT ORF to remove most of the CP-RT coding region; and iv) CP-RT Only, where the UAG stop codon was mutated to code for tyrosine G844C, so that only CP-RT was produced. In tests, only mutants CP-RT Only and No Protein accumulated in upper noninoculated leaves (Fig. 3B, middle panel, lanes 3 and 4). The other RNA-CP mutants analyzed failed to move systemically (Fig. 3B, middle panel, lanes 5, 7, 8, and 9).

Unexpectedly, the CP-RT Only mutant moved systemically and accumulated in upper noninoculated leaves (Fig. 3B, lane 3), inducing severe vein necrosis in *N. benthamiana* (Fig. 3C) compared with yellow mosaic symptoms induced by the wt virus (Fig. 3D). Western blotting with anti-CP antiserum revealed accumulation of CP-RT polypeptides but no CP subunits were detected in either inoculated or noninoculated upper leaves (Fig. 3E, lanes 1 and 2).

The RNA-CP mutants that were incapable of systemic movement all lacked subdomain 3 (SD3) of the CP-RT; it seems that RNA-CP cannot move systemically if CP-RT lacks the SD3 domain (mutants del-SD2&SD3 and del-3) or in the complete absence of CP-RT expression, although CP is expressed (mutants del-1, del-2, del-4, del-5, del-6, del-RT, and

CP Only). On the other hand, elimination of CP expression (mutants No Protein and CP-RT Only) enabled these mutants to move systemically in a nonvirion form, presumably as a viral ribonucleoprotein (vRNP) complex that includes RNA molecules and TGB1.

Deletions in the RT domain do not interfere with particle assembly.

It has been reported that the RT domain of BNYVV CP-RT plays a role in virus assembly (Schmitt et al. 1992) and the CP-RT of PMTV was detected at one end of some of the PMTV virions (Cowan et al. 1997). Therefore, by analogy with BNYVV, PMTV CP-RT may also be involved in initiation of virus particle assembly. To investigate this hypothesis, we performed immunosorbent electron microscopy (ISEM) experiments using anti-CP or anti-RT (antiserum against the C terminal region of RT protein) antibody preparations to determine whether PMTV particles were produced by any of the mutant clones. We observed many particles on grids that had been coated with anti-CP preparation and incubated with extracts from inoculated leaves, regardless of the presence of intact functional CP-RT in the mutants tested (Fig. 4; data not shown). As expected, in all experiments, fewer particles were trapped by the anti-RT than by the anti-CP, and no particles were observed in tests on inoculated or systemic leaves infected with the CP-RT Only mutant (data not shown). Therefore, both RT-null and C-terminally truncated mutants were competent in virus particle assembly but not the mutant that produced only the CP-RT polypeptide. Collectively, this data demonstrate that PMTV CP-RT is not needed for virion assembly.

PMTV CP-RT but not CP interacts with movement protein TGB1 in the yeast two-hybrid system.

PMTV virus particles were formed in the absence of functional CP-RT but were restricted to inoculated leaves; therefore, we hypothesized that CP-RT may function in systemic movement by interacting with other viral proteins (e.g., with move-

Fig. 4. Representative electron micrographs of *Potato mop-top virus* particles trapped from leaves inoculated with various RNA-CP mutants. Grids were coated with anti-CP immunoglobulin Gs. **A**, CP Only mutant; **B**, del-SD2 & SD3 mutant; **C**, del-SD2 mutant; **D**, wild-type virus. Bars represent 250 nm.

ment proteins). It has been shown previously that TGB1 self-interacts in the yeast two-hybrid system (Cowan et al. 2002). In this study, we used the yeast two-hybrid assay to investigate whether PMTV TGB1 interacts with CP and CP-RT. In parallel experiments, we also tested CP-CP-RT and CP-RT-CP-RT interactions, which can be predicted based on the structure and composition of the PMTV virions (Cowan et al. 1997). *Potato virus Y* HC-Pro was used as a positive control as it shows strong self-interactions (Cowan et al. 2002). Positive interactions were indicated by yeast growth in the absence of histidine and none of the protein fusions autoactivated histidine and uracil auxotrophy alone (data not shown). Cells transformed with plasmids encoding the binding domain pLex::CP-RT and activation domain pVP::TGB1 fusions grew in the absence of histidine, indicative of positive protein interactions (Fig. 5A, top left colonies within box). Similarly, as expected, cells co-transformed with pVP::CP-RT and either pLex::CP-RT or pLex::CP cassettes grew on medium lacking histidine (Fig. 5A, top left colonies within box). In this assay, TGB1 did not interact with CP. In addition, we

employed a quasi-quantitative β -galactosidase assay to measure the strength of the various PMTV protein interactions. In the interactions between CP-CP-RT and TGB1-TGB1, the blue β -galactosidase staining appeared quickly (in 2 h), indicative of strong interactions (Fig. 5A, left panel). The CP-RT self-interactions and CP-RT-TGB1 interactions were weaker, demonstrated by smaller colony size, slower growth on selective medium, and slower development of the β -galactosidase staining (which became darker after longer incubation) (Fig. 5A, middle panel). If the CP-RT self-interaction was mediated by the CP, then this weaker result may be explained by steric hindrance caused by the 89-kDa RT domain. These results established that PMTV TGB1 interacted with CP-RT but not with CP; therefore, we deduced that the interactions must be via the RT domain.

PMTV TGB1-CP-RT interaction is correlated with long-distance movement of RNA-CP.

The yeast two-hybrid assay was employed to determine whether the failure of CP-RT domain mutants to move long

Fig. 5. *Potato mop-top virus* (PMTV) CP-RT (readthrough of the coat protein gene) interactions. **A**, CP-RT interactions were assessed in yeast two-hybrid assays. Wild-type (wt) (black-box) or mutated PMTV sequences were fused to the LexA binding domain (cloned into pLex-Na plasmid) or VP16 DNA activation domain (cloned into pVP16+ plasmid). Transformants were selected for protein interactions on medium lacking uracil, histidine, leucine, and tryptophan (-UHLW). Medium lacking uracil, leucine, and tryptophan (-ULW) selected for the input plasmids only. β -Galactosidase activity was assessed by agarose overlay assays at two time points (at 2 and 16 h of incubation at 28°C). HC-Pro, self-interacting control protein. **B**, Detection of TGB1 associated with PMTV particles using anti-TGB1-, anti-RT-, or anti-CP-coated microtiter plates. Preimmune serum was used as control for anti-TGB1. Plant extracts were prepared from upper noninoculated leaves 21 dpi. Anti-CP monoclonal antibody mixtures SCR 68 and SCR 69 were used as detecting antibody. Absorbance values (absorbance at 405 nm) 1.5 times those of noninoculated healthy plants were considered as positives. The means ($n = 8$) of background absorbance from mock inoculation (white bars) served as the control to which the wt virus and the mutants were compared (color bars). Black bars are standard errors of the means. ELISA = enzyme-linked immunosorbent assay.

distance could be correlated with defects in protein interactions with TGB1. We were also interested to learn which part of the RT is involved in the interaction with TGB1. Three mutants were tested: mutant del-SD2, which moved systemically; mutant del-SD2&3, which did not move systemically; and mutant del-1, which possesses a short 129-amino-acid (aa) deletion at the C-terminus that rendered the protein unstable in plants.

Cells transformed with plasmids pVP::TGB1 and pLex::del-SD2 or pLex::del-1 grew in the absence of histidine, indicative of positive protein interactions (Fig. 5A). Likewise, cells co-transformed with plasmids pVP::del-SD2 and pLex::TGB1 grew on medium lacking histidine (Fig. 5A). The del-SD2&3 truncated protein failed to bind to TGB1, as shown by the lack of yeast growth (Fig. 5A). It should be noticed that del-1 truncated protein did bind to TGB1, although this interaction appeared weaker compared with wt CP-RT–TGB1 interactions, as revealed by β -galactosidase assay, smaller colony size, and slower growth (Fig. 5A). The stability of the del-1 protein in yeast cells compared with its rapid degradation in plant cells can be explained by translocation of the protein into the nucleus, where it is protected from degradation. The stability of the del-1 protein in yeast cells helped us to identify the “minimum interaction domain” required for efficient interaction of the CP-RT with TGB1. Collectively, these results indicated that a 33-aa region within the N-proximal part of the SD3 subdomain (Fig. 1, asterisk) is implicated in the interaction with TGB1. However, the strength of the del-1 and del-SD2 mutant interactions with TGB1 appeared less than the wt TGB1–CP-

RT interaction (according to β -galactosidase assay) (Fig. 5A), suggesting that both the SD2 subdomain and the C-proximal part of the SD3 subdomain probably also contribute to the efficient TGB1:CP-RT interaction. In addition, it should be noted that, although the interactions between TGB1 and CP-RT (del-SD2 mutant) were relatively weak compared with the wt, this mutant RNA could still move long distance in plants.

Detection of TGB1 in virus particles.

Because TGB1 interacts with CP-RT and CP-RT is present in virus particles, we wanted to establish whether TGB1 was also a component of virions. Experiments were done to determine whether TGB1 could be detected at one extremity of virus particles by immunogold EM following a method similar to that used by Cowan and associates (1997) to detect CP-RT at one extremity and employing an anti-TGB1 antiserum preparation. However, in these experiments, we failed to visualize significant immunogold staining of virus particle extremities. As an alternative approach, ELISA experiments were carried out with extracts from systemic leaves from plants inoculated with wt virus and RNA-CP mutants del-1, del-SD2&3, del-SD2, del-3, del-4, and CP-RT Only. The anti-TGB1, anti-RT, and anti-CP polyclonal preparations were used to coat ELISA plates, and anti-CP monoclonal antibodies (a mixture of monoclonal antibodies SCR 68 and SCR 69 specific for epitopes within the CP) were used for detection. This method did not detect free CP-RT expressed from the CP-RT Only mutant (Fig. 5B); therefore, we concluded that, in this test, the anti-CP monoclonal preparation did not detect the CP domain within the CP-RT fusion protein. This is probably because the epitopes detected by the two monoclonal antibodies were not available (Pereira et al. 1994). Two independent experiments using this approach showed that PMTV virions were detected in extracts prepared from upper noninoculated leaves of the plants inoculated with wt PMTV or del-SD2 regardless of the coating antiserum used. In other words, virus particles can be trapped using anti-TGB1 antiserum. These tests showed that TGB1 can be detected in virions. Moreover, all of the mutants (del-1, del-SD2&3, del-3, and del-4) in which RNA-CP did not move systemically but expressed TGB1 in systemic leaves (see RNA-TGB accumulation) (Figs. 2B, upper panel, lanes 4, 5, and 8 through 10; 3B, lower panel, lanes 3 and 5 through 7) served as appropriate negative controls in these experiments.

Fig. 6. Mutant rescue. A, Reverse-transcription polymerase chain reaction (PCR) of samples prepared from inoculated and upper noninoculated *Nicotiana benthamiana* leaves of plants infected with various RNA-CP mutants and wild-type (wt) *Potato mop-top virus* (PMTV). The nucleotide length of marker DNAs are shown on the right. The type of PMTV RNA detected by reverse-transcription PCR is indicated on the left. B and C, Appearance of PMTV symptoms in *N. benthamiana* infected with B, Tyr-del-SD2 mutant and C, Tyr-del-SD2&3 mutant.

Fig. 7. Schematic diagram of the predicted composition of the transport forms of wt and mutant *Potato mop-top virus* and their localizations.

Rescue of movement defective CP-RT mutant RNAs by blocking CP expression.

The finding that the No Protein mutant RNA moved systemically in a nonvirion form (Fig. 3B, lane 4) whereas the mutant that only produced CP did not suggests that CP expressed in the absence of a functional CP-RT (that interacts with TGB1) inhibits long-distance movement of RNA-CP (and mutant RNAs). To test this idea, the point mutation (G844C) was introduced into the movement-deficient mutants del-SD2&3 and del-1 and into the del-SD2 mutant to block expression of the CP subunits as described before, with the expectation that the mutant RNAs would then move systemically in a nonvirion form. These mutants, named Tyr-del-SD2&3, Tyr-del-1, and Tyr-del-SD2, were compared with the parental mutants for their ability to move systemically. The results are shown in Figure 6. As expected, RNA 1 was detected in all samples from upper noninoculated (systemic) leaves (Fig. 6A, middle panel) and RNA-CP was readily detected in upper leaves of all plants inoculated with the G844C mutants. Moreover, Tyr-del-SD2&3 and Tyr-del-SD2 mutants (but not Tyr-del-1) induced severe vein necrosis in *N. benthamiana* similar to previously characterized mutant CP-RT Only (Fig. 6B). Therefore, these experiments showed that the RNA-CP mutants that were defective in systemic movement could be rescued by preventing expression of CP subunits.

DISCUSSION

Two stages of virus systemic movement have been distinguished, cell-to-cell and long-distance each with different requirements (Lucas 2006; Morozov and Solovyev 2003; Taliansky and Torrance 2008; Taliansky et al. 2008). A common transport pathway utilized by plant viruses for long-distance movement is the vascular system and, in particular, phloem transport of macromolecules. Although progress is being made in understanding the intracellular and cell-to-cell movement of PMTV (and other viruses with TGB), little is known about the virus long-distance movement mechanisms. PMTV is unusual in that it is one of a small number of viruses, including the umbraviruses (Taliansky and Robinson 2003), *Barley stripe mosaic virus* (Petty and Jackson 1990), *Tobacco rattle virus* (Harrison and Robinson 1986), and *Brome mosaic virus* (Gopinath and Kao 2007), whose genomic RNA can move independently of CP (McGeachy and Barker 2000; Savenkov et al. 2003). Most of these viruses have segmented genomes and it has been suggested that the capacity for independent long-distance RNA trafficking may be necessary to reconstitute a viable infection if the genome segments become separated (Gopinath and Kao 2007). However, the long-distance movement of RNA1 and RNA-TGB in the absence of RNA-CP may have adverse consequences for PMTV, resulting in the loss of genome segments and extinction of the population, because it has been shown that the CP-RT is needed for transmission by the soilborne vector *S. subterranea* (Reavy et al. 1998).

In a previous study, we demonstrated that PMTV RNA-CP is not needed for long-distance movement of the other two RNAs but is required for symptom induction in *N. benthamiana* (Savenkov et al. 2003). In this article, the role of the CP and CP-RT in long-distance movement of RNA-CP was examined. Mutations were introduced into an RNA-CP clone within the ORF encoding the CP and CP-RT proteins to examine their effect on RNA-CP replication, accumulation in inoculated leaves, and long-distance movement. The results showed that expression of mutant clones expressing only CP or CP-RT with deletions in the RT region inhibited the long-distance movement of the RNA-CP in *N. benthamiana* but not the

movement of the other two RNA species. Amino acids in the C-terminal segment of the RT domain between 2,300 and 2,730 nt (spanning the subdomain SD3) were shown to be required for long-distance movement. In contrast, deletions between nucleotides 1,756 and 2,299 (within the SD2 subdomain) did not inhibit movement, and isolates carrying these deletions have been found in nature as well as in cultures established after successive manual passage (Sandgren et al. 2001). Deletion of the SD2 subdomain in the CP-RT resulted in milder symptoms, suggesting that SD2 might be a virulence determinant. On the other hand, infections with mutant CP-RT clones in which the CP expression was blocked allowed long-distance movement of the RNA and induced severe necrotization of veins, suggesting that overexpressed CP-RT may interfere with proper functioning of the phloem, perhaps triggering a localized ectopic programmed cell death. The fact that PMTV CP-RT Only mutants were viable and moved long distance in plants was an unexpected result because a similar mutant (conversion of the suppressible UAG to the tyrosine codon UAC) in BNYVV was not viable and it did not accumulate even in the inoculated leaves (Schmitt et al. 1992).

A nontranslatable RNA-CP mutant was found to move systemically and the long-distance movement of the CP-RT mutants could be rescued by preventing the expression of CP subunits. We conclude that, in the absence of CP, the RNA-CP moves as a vRNP complex similar to the other RNA species. The actual composition of PMTV RNP complexes is unknown but, by analogy with other hordei-like TGB-encoding viruses, it is most likely to include TGB1 (Lim et al. 2008). Expression of CP in the absence of CP-RT (or together with deleted CP-RT) inhibited RNA-CP long-distance movement but did not affect the movement of the other two RNAs. EM analysis of inoculated leaves showed that virus particles were present and, therefore, CP-RT is not required for particle assembly, but it is not clear whether only the RNA-CP molecules were encapsidated; furthermore, these results raise the question of why only RNA-CP movement was inhibited. A possible explanation may lie in sequence differences found in the UTR of the RNAs; the 3' ends of all three PMTV RNA species are highly conserved and form a tuna-like structure. However, at the 5' end, although there is good alignment of the first 42 nt between RNA1 and RNA-TGB, the alignment with RNA-CP is extremely weak (Savenkov et al. 1999). It is possible that the 5' end of the RNA-CP contains a nucleating element which preferentially binds to the CP subunits compared with RNA1 and RNA-TGB origins of assembly. Further work is needed to investigate the role of 5' ends in particle assembly, the relative binding affinities of CP and TGB1, and the packaging capacity of the different RNA species.

Analysis of protein interactions in a yeast two-hybrid assay revealed self-interactions between CP-RT and TGB1. In addition, heterologous interactions were observed between CP-RT and TGB1 and CP-RT and CP but not between CP and TGB1. Analysis of CP-RT mutants indicated that mutations that were defective in RNA-CP systemic movement were also defective in interactions with TGB1. A short, 33-aa region within the N-terminal part of the SD3 subdomain of CP-RT was implicated in the interaction with TGB1.

A diagram summarizing our results and presenting a model for the mechanism of RNA-CP movement is presented in Figure 7. The results show that all three RNAs can move as vRNP presumably containing TGB1 without any requirement for CP. In addition, only RNA-CP molecules expressing CP together with CP-RT protein capable of interacting with TGB1 can move long distance. RNA-CP molecules expressing CP alone (or CP with CP-RT mutants that do not interact with TGB1) do not move to upper leaves but accumulate in inoculated leaves,

and virus particles are formed. CP-RT is not needed for particle assembly and mutants expressing only CP-RT do not form particles. Mutant CP-RT does not inhibit movement when expressed alone (in the absence of CP), indicating that the CP-RT does not function in movement unless incorporated into particles.

ELISA data suggests that CP-RT and TGB1 are associated with virus particles. Previously, we showed that CP-RT domain was present in a small proportion of virions at one extremity (Cowan et al. 1997); therefore, these two observations led to the hypothesis that the RNA-CP molecules move long distance as assembled particles containing CP-RT and TGB1, possibly incorporated at the tip. However, this needs further investigation because it is also possible that TGB1 associates with CP-RT in some other way to facilitate movement. RNA-CP mutants are capable of cell-to-cell movement and accumulate in the inoculated leaves but it is not clear whether they move as vRNP complexes or assembled particles. It is possible that the particles observed in the mutant cultures are not competent for movement and remain confined to the cells in which CP is expressed with all three RNAs moving as vRNP complexes; however, if this is so, then we might expect both the mutant and wt RNA-CP to continue to move as vRNP with no barrier to long-distance movement (as occurs when CP expression is suppressed). The most plausible explanation is that, in the presence of CP, RNA-CP moves cell-to-cell as particles but only the hypothesized 'tipped' particles containing TGB1 can enter the vasculature for long-distance movement.

EM observations of thin sections of PMTV-infected *N. benthamiana* leaves have revealed the presence of PMTV particles in all cell types, including epidermal, mesophyll, and vascular (phloem and xylem parenchyma and sieve elements) tissues (Lioliopoulou 2001). A similar EM study of leaf sections infected with mutant RNA-CP clones is needed to establish the localization of virus particles and whether they enter the vasculature.

In *Potato virus X*, a monopartite virus with filamentous particles that also encodes a TGB movement module, the TGB1 is multifunctional (Verchot-Lubicz et al. 2007). In addition to promoting virus movement, it was shown to suppress RNA silencing (Voinnet et al. 2000) and to be attached to one extremity of virus particles where it is thought to promote particle destabilization and RNA translation (Kiselyova et al. 2003; Rodionova et al. 2003); it was suggested that this interaction at the particle extremity might also facilitate cell-to-cell movement (Karpova et al. 2006). It is possible that PMTV TGB1 might play a similar role in translation or silencing. The experiments reported in this article show that TGB1 interacts with the CP-RT domain, the interaction is correlated with long-distance movement of RNA-CP, and both proteins can be detected in assembled particles. However, the observations that RNA-CP mutants expressing CP and CP-RT mutant proteins that do not interact with TGB1 can form particles, accumulate, and spread cell to cell show that an interaction between CP-RT and TGB1 is not necessary for RNA translation and replication (at least in cell-to-cell spread). Furthermore, experiments using the patch agroinfiltration assay (Johansen and Carrington 2001) to determine whether PMTV TGB1 is a suppressor of silencing failed to reveal such a function (E. I. Savenkov, unpublished data). Therefore, the data suggest that the two TGB1 proteins have somewhat different functions.

Our data showing that RNA-1 and RNA-TGB can move independently of RNA-CP in *N. benthamiana* can explain observations of patchy distribution in potato (the natural host) where PMTV does not spread to all plants grown from infected tubers (Arlı Sokmen et al. 1998). Often, the viral CP antigen can be detected by serological methods in some leaves

and auxiliary shoots of the infected potato plants but not in others of the same plant (Torrance 1992) and RNA-TGB could be detected by reverse-transcription PCR methods in some "virus-free" (CP-antigen free) auxiliary shoots (Arlı Sokmen et al. 1998). It is possible that, in natural infections, some RNA-1 and RNA-TGB molecules form RNP complexes rather than particles and move long distance, establishing infections in the absence of RNA-CP. Indeed, it was shown that RNA 1 and RNA-TGB could move long distance whereas RNA-CP was not detected in *N. benthamiana* plants transgenic for PMTV CP gene (McGeachy and Barker 2000). Moreover, when RNA-CP-free extracts from those plants were inoculated onto nontransgenic *N. benthamiana* and *N. clevelandii*, RNA 1 and RNA-TGB accumulated in upper noninoculated leaves (McGeachy and Barker 2000). Independent movement of RNA 1 and RNA-TGB was confirmed using direct inoculation of RNA 1 and RNA-TGB transcripts onto *N. benthamiana* (Savenkov et al. 2003). Therefore, it seems that, in at least three plant species (*Solanum tuberosum*, *N. benthamiana*, and *N. clevelandii*), the RNA-CP is not necessary for the long-distance movement of the other two RNAs.

In summary, the results reveal an unusual feature of PMTV infections: namely, that the genomic RNAs have different requirements for long-distance movement. In the absence of CP, all three genomic RNAs can move systemically as RNP. In the presence of CP, RNA-CP (and possibly the other RNA species) is encapsidated in particles associated with CP-RT and TGB1. However, in mutant clones expressing only CP or mutations in the CP-RT that inhibit association with TGB1, the systemic movement of RNA-CP but not the other two genomic RNAs is inhibited.

MATERIALS AND METHODS

Construction of plasmids.

Standard recombinant DNA procedures were performed by use of a combination of PCR, site-directed mutagenesis, and swapping of restriction fragments. The plasmid pPMTV-2 (Savenkov et al. 2003) was used to generate the mutants and constructs described in this study. First, the del-1 construct was engineered by ligating *Bgl*II/*Apa*I-digested PCR product generated with primers PMTV-RNA2-*Bgl*II-pos and PMTV-RNA2-del-1 (Supplementary Table 1), and *Apa*I/*Mlu*I-digested PCR product obtained with primers PMTV-RNA2-*Apa*I-pos and RNA2-*Mlu*I-*Xba*I into *Bgl*II/*Mlu*I-digested pPMTV-2. To obtain del-2, del-3, del-4, del-5, del-6, and del-RT, the *Bgl*II/*Apa*I fragment in del-1 was swapped for *Bgl*II/*Apa*I-digested PCR products generated with primers PMTV-RNA2-*Bgl*II-pos and PMTV-RNA2-del-2, PMTV-RNA2-del-3, PMTV-RNA2-del-4, PMTV-RNA2-del-5, PMTV-RNA2-del-6, or PMTV-RNA2-delRT, respectively.

To construct No Protein mutant, ochre and amber termination codons were introduced at the start of the CP ORF via two PCR assays from pPMTV-2 template DNA with primers pUC-M13F and PMTV-CP-*Nco*I-del, and PMTV-CP-*Nco*I-stop-stop and pUC-M13R. The *Sph*I/*Nco*I-digested PCR product, encompassing PMTV RNA2 5' UTR, and *Nco*I/*Mlu*I-digested PCR product were ligated into *Sph*I/*Mlu*I-digested pPMTV-2. CP-RT Only mutant was engineered by ligating *Bgl*II/*Nar*I-digested PCR product generated with primers PMTV-RNA2-*Bgl*II-pos and CP-Tyr-RT-anti-sense-Nar, and *Nar*I/*Mlu*I-digested PCR product obtained with primers CP-Tyr-RT-sense-Nar and RNA2-*Mlu*I-*Xba*I, into *Bgl*II/*Mlu*I-digested pPMTV-2. To obtain CP Only mutant, the *Nar*I/*Eco*RV fragment in CP-RT Only was swapped for *Nar*I/*Eco*RV-digested PCR products generated with primers CP-*Nar*I-stop-stop and pUC-M13R.

Del-SD2 mutant was engineered by ligating *Bgl*III/*Aat*II-digested PCR product generated with primers PMTV-RNA2-*Bgl*II-pos and PMTV-*Aat*II-*Apa*I-center-neg, and *Aat*II/*Mlu*I-digested PCR product obtained with primers PMTV-*Aat*II-center-pos and RNA2-*Mlu*I-*Xba*I into *Bgl*III/*Mlu*I-digested pPMTV-2. Del-SD2&3 mutant was constructed by ligating *Bgl*III/*Apa*I-digested PCR product generated with primers PMTV-RNA2-*Bgl*II-pos and PMTV-*Aat*II-*Apa*I-center-neg, and *Apa*I/*Mlu*I-digested PCR product obtained with primers PMTV-RNA2-*Apa*I-pos and RNA2-*Mlu*I-*Xba*I into *Bgl*III/*Mlu*I-digested pPMTV-2.

To construct Tyr-del-SD2, Tyr-del-SD2&3, and Tyr-del-1, the *Bgl*III/*Nhe*I fragments, encompassing CP ORF and part of RT ORF, in del-SD2, del-SD2&3, and del-1 were swapped for the corresponding *Bgl*III/*Nhe*I fragment from CP-RT Only mutant.

Plant inoculations and analyses.

Inoculation of *N. benthamiana* plants with recombinant viruses, Western blot analyses, and ELISA were carried out as described (Savenkov et al. 2003; Yelina et al. 2002). Entire upper leaves (fifth and sixth above the inoculated) were sampled 14 dpi from 1-month-old plants and 28 dpi from 1.5-month-old plants.

Northern blot analysis.

Total RNA was isolated using a Qiagen (Hilden, Germany) RNA minikit 14 dpi. Heat-denatured RNA (7 µg/lane) was separated by electrophoresis on 1.2% agarose gels and transferred to Hybond membranes (AmershamBiosciences, Little Chalfont, U.K.). Antisense [³²P]UTP-labeled RNA probes were generated by in vitro transcription with T7 or SP6 RNA polymerase (Promega Corp., Madison, WI, U.S.A.) from *Nor*I-linearized pGEM5-tRNA-like (PMTV tRNA-like structure probe) or *Xba*I-digested pCRII-topo-p404 (RNA2 5' probe, 5' rapid amplification of cDNA ends RNA2 650-nt PCR product cloned into pCRII-topo plasmid) (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. Prehybridization and hybridization were carried out for 1 h and overnight, respectively, at 65°C. The membranes were washed at 70°C twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 15 min each wash, in 2× SSC and 0.1% sodium dodecyl sulfate (SDS) for 30 min, and twice in 0.1× SSC and 0.1% SDS for 15 min each wash. After the final washing, the membranes were wrapped in polyethylene plastic and placed together with X-ray film in an exposure cassette (Molecular Dynamics, Sunnyvale, CA, U.S.A.) for 1 to 24 h. The film was then scanned with a Molecular Imager FX and the signal strength was quantified with Quantity One software (Bio-Rad).

ISEM and ELISA.

Antisera used were preparations of anti-RT and anti-CP polyclonal antibodies and anti-CP monoclonal antibodies (a mixture of SCR 68 and SCR 69) (Pereira et al. 1994; Torrance et al. 1993). For ISEM, anti-RT and anti-CP polyclonal were used at approximately 1 µg/ml to trap PMTV particles from leaf extracts and the experiments were done essentially as described (Cowan et al. 1997). For ELISA, anti-RT or anti-CP polyclonal preparations were used to coat microtiter plates and the anti-CP monoclonal followed by anti-mouse alkaline phosphatase conjugate (Sigma, St. Louis) were used for detection essentially as described (Torrance 1992).

Construction of clones for yeast two-hybrid assays.

Yeast expression cassettes were generated using the pLex-Na and pVP16+ vectors (Hollenberg et al. 1995). The minor

CP sequence was amplified with primers *Eco*RI-N-ter-CP and *Pst*I-C-ter-RT, or with primers *Apa*I-N-ter-CP and *Eco*RI-C-ter-RT using Phusion Taq polymerase (Finnzymes) and CP-RT Only as a template. The PCR products were digested with *Eco*RI/*Pst*I or with *Apa*I/*Eco*RI, and cloned into the same sites of pLex-Na or pVP16+ to give pLex-CP-RT or pVP16-CP-RT.

The mutated minor CP sequences were amplified with *Eco*RI-N-ter-CP and *Pst*I-C-ter-RT, or with primers *Apa*I-N-ter-CP and *Eco*RI-C-ter-RT using Phusion Taq polymerase (Finnzymes) and Tyr-del-1 or Tyr-del-SD2 or Tyr-del-SD2&3 as templates. The PCR products were digested with *Eco*RI/*Pst*I or with *Apa*I/*Eco*RI, and cloned into the same sites of pLex-Na or pVP16+ to give pLex-del-1 or pVP16-del-1 or pLex-del-SD2 or pVP16-del-SD2 or pLex-del-SD2&3 or pVP16-del-SD2&3. pLex-CP, pLex-TGB1, pVP16-TGB1, pLex-HC-Pro, and pVP16-HC-Pro constructs were described previously (Cowan et al. 2002).

Yeast two-hybrid assays.

The interaction between Lex A and VP16 fusion proteins was evaluated in *Saccharomyces cerevisiae* strain L40 as described (Cowan et al. 2002).

Reverse-transcription PCR.

Total RNA was extracted from *N. benthamiana* leaves using the RNeasy plant minikit (Qiagen) following the manufacturer's instructions. DNase I-treated total RNA (1 µg) was then used in a reverse-transcription reaction with M-MuLV reverse transcriptase (Promega Corp.) and with either POL R or CP R primer. The CP-gene specific primers CP F and CP R were used to detect RNA-CP by PCR. The replicase-specific POL F and POL R were used to detect RNA 1.

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LITERATURE CITED

- Arli Sokmen, M., Barker, H., and Torrance, L. 1998. Factors affecting the detection of potato mop-top virus in potato tubers and improvements of test procedures for more reliable assays. *Ann. Appl. Biol.* 133:55-63.
- Cowan, G. H., Torrance, L., and Reavy, B. 1997. Detection of potato mop-top virus capsid readthrough protein in virus particles. *J. Gen. Virol.* 78:1779-1783.
- Cowan, G. H., Lioliopoulou, F., Ziegler, A., and Torrance, L. 2002. Sub-cellular localisation, protein interactions and RNA binding of *Potato mop-top virus* triple gene block proteins. *Virology* 298:106-115.
- Gopinath, K., and Kao, C. C. 2007. Replication-independent long-distance trafficking by viral RNAs in *Nicotiana benthamiana*. *Plant Cell* 19:1179-1191.
- Harrison, B. D., and Robinson, D. J. 1986. Tobravirus. Pages 339-369 in: *The Plant Viruses*, vol. 2, *The Rod-shaped Plant Viruses*. M. H. V. Van Regenmortel and H. Fraenkel-Conrat, eds. Plenum, New York.
- Haupt, S., Cowan, G. H., Ziegler, A., Roberts, A. G., Oparka, K. J., and Torrance, L. 2005. Two plant-viral movement proteins traffic in the endocytic recycling pathway. *Plant Cell* 17:164-181.
- Hollenberg, S. M., Sternglanz, R., Cheng, P. F., and Weintraub, H. 1995. Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol. Cell. Biol.* 15:3813-3822.
- Johansen, L. K., and Carrington, J. C. 2001. Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiol.* 126:930-938.
- Karpova, O. V., Zayakina, O. V., Arkhipenko, M. V., Sheval, E. V., Kiselyova, O. I., Poljakov, V. Y., Yaminsky, I. V., Rodionova, N. P., and Atabekov, J. G. 2006. *Potato virus X* RNA-mediated assembly of sin-

- gle-tailed ternary 'coat protein-RNA-movement protein' complexes. *J. Gen. Virol.* 87:2731-2740.
- Kashiwazaki, S., Scott, K. P., Reavy, B., and Harrison, B. D. 1995. Sequence analysis and gene content of potato mop-top virus RNA 3: Further evidence of heterogeneity in the genome organization of furoviruses. *Virology* 206:701-706.
- Kiselyova, O. I., Yaminsky, I. V., Karpova, O. V., Rodionova, N. P., Kozlovsky, S. V., Arkhipenko, M. V., and Atabekov, J. G. 2003. AFM study of *Potato virus X* disassembly induced by movement protein. *J. Mol. Biol.* 332:321-325.
- Koenig, R., and Lesemann, D. E. 2005. Genus *Pomovirus*. In: *Virus Taxonomy, Eighth Rep. Int. Committee on Taxonomy of Viruses*. M. H. V. van Regenmortel, C. Fauquet, and D. H. L. Bishop, eds. Harcourt International, Boston.
- Lim, H.-S., Bragg, J. N., Ganesan, U., Lawrence, D. M., Yu, J., Isogai, M., Hammond, J., and Jackson, A.O. 2008. Triple gene block protein interactions involved in movement of *Barley stripe mosaic virus*. *J. Virol.* 82:4991-5006.
- Lioliopoulou, F. 2001. Studies on the localization and function of *Potato mop-top virus* proteins. Ph.D. thesis, University of Dundee, U.K.
- Lucas, W. J. 2006. Plant viral movement proteins: Agents for cell-to-cell trafficking of viral genomes. *Virology* 344:169-184.
- McGeachy, K. D., and Barker, H. 2000. *Potato mop-top virus* RNA can move long-distance in the absence of coat protein: Evidence from resistant, transgenic plants. *Mol. Plant-Microbe Interact.* 13:125-128.
- Morozov, S. Yu., and Solovyev, A. G. 2003. Triple gene block: Modular design of a multifunctional machine for plant virus movement. *J. Gen. Virol.* 84:1351-1366.
- Pereira, L. G., Torrance, L., Roberts, I. M., and Harrison, B. D. 1994. Antigenic structure of the coat protein of potato mop-top furovirus. *Virology* 203:277-285.
- Petty, I. T., and Jackson, A. O. 1990. Mutational analysis of barley stripe mosaic virus RNA beta. *Virology* 179:712-718.
- Reavy, B., Arif, M., Cowan, G. H., and Torrance, L. 1998. Association of sequences in the coat protein/readthrough domain of potato mop-top virus with transmission by *Spongospora subterranea*. *J. Gen. Virol.* 79:2343-2347.
- Rodionova, N. P., Karpova, O. V., Kozlovsky, S. V., Zayakina, O. V., Arkhipenko, M. V., and Atabekov, J. G. 2003. Linear remodeling of helical virus by movement protein binding. *J. Mol. Biol.* 333:565-572.
- Sandgren, M., Savenkov, E. I., and Valkonen, J. P. T. 2001. The readthrough region of *Potato mop-top virus* (PMTV) coat protein encoding RNA, the second largest RNA of PMTV genome, undergoes structural changes in naturally infected and experimentally inoculated plants. *Arch. Virol.* 146:467-477.
- Savenkov, E. I. 2002. Genus *Pomovirus*. Pages 1297-1301 in: *The Springer Index of Viruses*. C. A. Tidona and G. Darai, eds. Springer-Verlag, Heidelberg, Germany.
- Savenkov, E. I., Sandgren, M., and Valkonen, J. P. T. 1999. Complete sequence of RNA 1 and the presence of tRNA-like structures in all RNAs of Potato mop-top virus, genus *Pomovirus*. *J. Gen. Virol.* 80:2779-2784.
- Savenkov, E. I., Germundsson, A., Zamyatnin, A. A., Jr., Sandgren, M., and Valkonen, J. P. T. 2003. *Potato mop-top virus*: The coat protein-encoding RNA and the gene for cysteine-rich protein are dispensable for systemic virus movement in *Nicotiana benthamiana*. *J. Gen. Virol.* 84:1001-1005.
- Schmitt, C., Balmori, E., Jonard, G., Richards, K. E., and Guillely, H. 1992. In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: Evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. *Proc. Natl. Acad. Sci. U.S.A.* 89:5715-5719.
- Scott, K. P., Kashiwazaki, S., Reavy, B., and Harrison, B. D. 1994. The nucleotide sequence of potato mop-top virus RNA 2: A novel type of genome organisation for a furovirus. *J. Gen. Virol.* 75:3561-3568.
- Taliansky, M. E., and Robinson, D. J. 2003. Molecular biology of umbraviruses: Phantom warriors. *J. Gen. Virol.* 84:1951-1960.
- Taliansky, M., and Torrance, L. 2008. Plant virus movement. In: *Encyclopedia of Life Sciences*. John Wiley & Sons, Ltd., Chichester, U.K.
- Taliansky, M., Torrance, L., and Kalinina, N. O. 2008. Role of plant virus movement proteins. In: *Methods in Molecular Biology*, vol. 451, *Plant Virology Protocols: From Viral Sequence to Protein Function*. G. D. Foster, I. E. Johansen, Y. Hong, and P. D. Nagy, eds. Humana Press, Totowa, NJ, U.S.A.
- Torrance, L. 1992. Serological methods to detect plant viruses: Production and use of monoclonal antibodies. In: *Techniques for the Rapid Detection of Plant Pathogens*. J. M. Duncan and L. Torrance, eds. Blackwell Scientific Publications, Oxford.
- Torrance, L. *Pomovirus*. 2008. In: *Encyclopedia of Virology*, third ed. B. W. H. Mahy and M. H. V. van Regenmortel, eds. Elsevier, Amsterdam.
- Torrance, L., Cowan, G. H., and Pereira, L. G. 1993. Monoclonal antibodies specific for potato mop-top virus, and a revised estimate of coat protein molecular weight. *Ann. Appl. Biol.* 122:311-322.
- Torrance, L., Cowan, G. H., Arli-Sokmen, M., and Reavy, B. 1999. A naturally occurring deleted form of RNA 2 of *Potato mop-top virus*. *J. Gen. Virol.* 80:2211-2215.
- Verchot-Lubicz, J., Ye, C.-M., and Bamunusinghe, D. 2007. Molecular biology of potexviruses: Recent advances. *J. Gen. Virol.* 88:1643-1655.
- Voinnet, O., Lederer, C., and Baulcombe, D. C. 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. *Cell* 103:157-167.
- Yelina, N. E., Savenkov, E. I., Solovyev, A. G., Morozov, S. Yu., and Valkonen, J. P. T. 2002. Long-distance movement, virulence, and RNA silencing suppression controlled by a single protein in Hordei- and Potyviruses: Complementary functions between virus families. *J. Virol.* 76:12981-12991.
- Zamyatnin, A. A., Jr., Solovyev, A. G., Savenkov, E. I., Germundsson, A., Sandgren, M., Valkonen, J. P. T., and Morozov, S. Yu. 2004. Transient co-expression of individual genes encoded by the triple gene block of *Potato mop-top virus* reveals requirements for TGBp1 trafficking. *Mol. Plant-Microbe Interact.* 17:921-930.