Available online at www.sciencedirect.com
SCIENCE DIRECT
Virology 351 (2006) 455–465
VIROLOGY
www.elsevier.com/locate/yviro

Functional analysis of the 5′ untranslated region of potexvirus RNA reveals a role in viral replication and cell-to-cell movement

Tony J. Lough [a,*], Robyn H. Lee [a], Sarah J. Emerson [a], Richard L.S. Forster [a], William J. Lucas [b]

[a] *Horticulture and Food Research Institute of New Zealand, Plant Health and Development Group, Private Bag 11030, Palmerston North, New Zealand*
[b] *Section of Plant Biology, Division of Biological Sciences, University of California, Davis, CA 95616, USA*

Received 17 February 2006; returned to author for revision 6 March 2006; accepted 27 March 2006
Available online 11 May 2006

Abstract

Cell-to-cell movement of potexviruses requires cognate recognition between the viral RNA, the triple gene block proteins (TGBp1–3) and the coat protein (CP). *cis*-acting motifs required for recognition and translocation of viral RNA were identified using an artificial potexvirus defective RNA encoding a green fluorescent protein (*GFP*) reporter transcriptionally fused to the terminal viral sequences. Analysis of GFP fluorescence produced in vivo from these defective RNA constructs, referred to as chimeric RNA reporters, was used to identify viral *cis*-acting motifs required for RNA trafficking. Mapping experiments localized the *cis*-acting element to nucleotides 1–107 of the *Potato virus* X (PVX) genome. This sequence forms an RNA secondary structural element that has also been implicated in viral plus-strand accumulation [Miller, E.D., Plante, C.A., Kim, K.-H., Brown, J.W. and Hemenway, C. (1998) *J. Mol. Biol.* 284, 591–608]. While replication and movement functions associated with this region have not been separated, these results are consistent with sequence-specific recognition of RNA by the viral movement protein(s). This situation is unusual among viral movement proteins that typically function to translocate RNA between cells in a non-sequence-specific manner. These data support the concept of *cis*-acting elements specifying intercellular potexvirus RNA movement and thus provide a basis for dissection of RNA-mediated intercellular communication in plants.

Keywords: Cell-to-cell movement; Plasmodesmata; Potexvirus; Triple gene block; Defective RNA

Introduction

Asymmetric distribution of mRNA within cells is controlled by protein–RNA interactions. This process is mediated by nucleotide-specific *cis*-acting elements that control subcellular delivery and hence localized protein synthesis (Bassell and Kelic, 2004; Bassell and Twiss, 2006). This process has been shown to underlie a range of cellular and developmental events (Choi et al., 2000; Miki et al., 2005). A new twist to RNA function is in its emerging role in control of aberrant RNA and virus/transposon challenge (Bartel, 2004; Lindbo and Dougherty, 2005; Zamore and Haley, 2005). Here, non-cell-autonomous nucleotide-specific molecules mediate targeted RNA degradation, via RNA interference (Fire et al., 1998; Meins et al., 2005; Napoli et al., 1990; Zamore and Haley, 2005). In plants, cell-to-cell spread of the silencing signal occurs through plasmodesmata (PD), membrane-lined intercellular channels that provide a pathway for regulated movement of macromolecules (Lough and Lucas, 2006; Oparka, 2004; Mlotshwa et al., 2002; Voinnet et al., 1998).

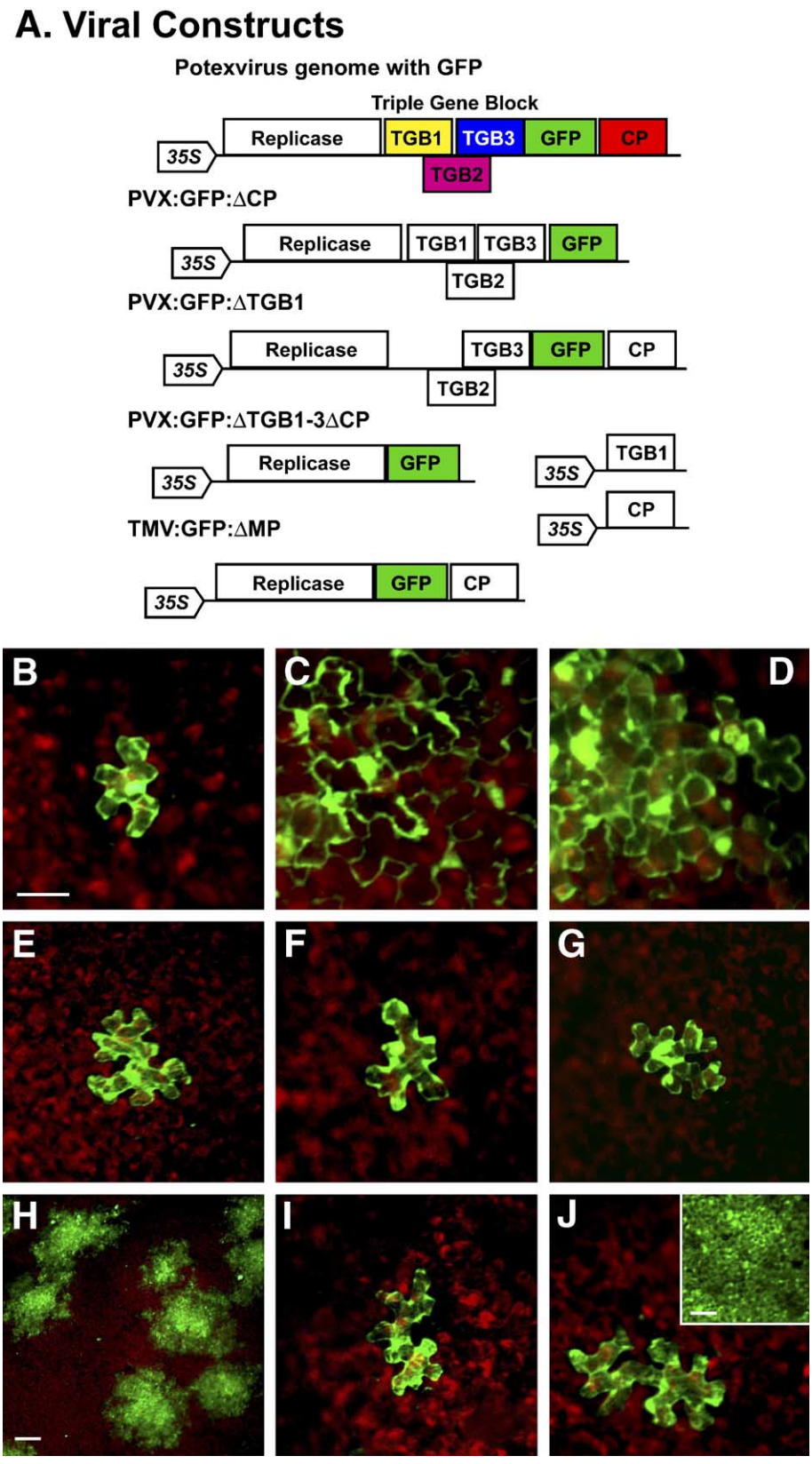
Pioneering genetic, molecular and cellular studies on plant virus movement afforded important insights into the trafficking of proteins and nucleic acid complexes within the body of the plant. Most plant viruses encode one or more movement proteins (MPs) that are required for cell-to-cell transport of the infectious nucleic acid(s) (Lazarowitz and Beachy, 1999; Lucas, 2006; Waigmann et al., 2004). The plant vascular system, and specifically the phloem, serves as the conduit for systemic spread of viral nucleic acids and the viral-directed silencing signal(s) (Lough and Lucas, 2006; Lucas, 2006; Mlotshwa et al., 2002; Voinnet, 2005; Yoo et al., 2004). An emerging paradigm suggests a further role for the phloem in mediating

* Corresponding author. Current address: Genesis Research and Development Corporation, 1 Fox St., PO Box 50, Auckland, New Zealand. Fax: +64 9 373 2189.
E-mail address: t.lough@genesis.co.nz (T.J. Lough).

0042-6822/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.03.043

inter-organ delivery of specific transcripts (Ruiz-Medrano et al., 1999; Xoconostle-Cázares et al., 1999). This concept is supported by the finding that phloem-mobile transcripts such as *Me* (Kim et al., 2001), *GAIP* (Haywood et al., 2005) and *FT* (Huang et al., 2005) influence developmental processes in distant tissues.

Although much remains to be learned about the mechanism(s) that underlies selective trafficking of proteins and ribo-



nucleoprotein (RNP) complexes through PD, there are indications that this process involves structural motifs associated with non-cell-autonomous proteins (Aoki et al., 2002; Kim et al., 2005). Furthermore, studies performed on *Potato spindle tuber viroid* (PSTVd) have established that specific RNA sequences are required for viroid cell-to-cell movement (Qi et al., 2004; Zhu et al., 2001, 2002). Interestingly, possession of a defined sequence motif is sufficient for the PSTVd^{NB} strain to traffic from the phloem into mesophyll cells, whereas strain PSTVd^{NT} remains confined to the phloem. Viroid movement was found to be both unidirectional and developmentally regulated (Qi et al., 2004; Zhu et al., 2001, 2002), consistent with a requirement for a specific interaction with the host cellular machinery.

Insight into the complexity of viral RNA movement has also been gained by studies of the potexviruses. These viruses employ a complex cell-to-cell movement strategy involving three proteins translated from overlapping open reading frames (ORFs), termed the triple gene block (TGB). (The TGB ORFs and proteins will be referred to as TGB1–3 and TGBp1–3, respectively.) TGBp1 has been defined as the potexvirus MP, based on its capacity to: (a) bind viral RNA; (b) increase the PD size exclusion limit; (c) traffic itself through PD and (d) potentiate the cell-to-cell movement of viral RNA in the presence of TGBp2 and TGBp3 (Beck et al., 1991; Lough et al., 1998, 2000; Morozov and Solovyev, 2003; Oparka et al., 1996; Tamai and Meshi, 2001; Verchot-Lubicz, 2005; Yang et al., 2000). A fourth potexvirus protein, the coat protein (CP), is also required for potexvirus cell-to-cell movement (Baulcombe et al., 1995; Chapman et al., 1992; Forster et al., 1992). The TGBp1–RNA complex appears to be delivered to PD by means of vesicle trafficking along the ER-microfilament pathway (Lucas, 2006; Oparka, 2004). In this model, TGBp2 and TGBp3 are integral membrane proteins that serve to anchor the TGBp1–RNA complex to the vesicle surface (Haupt et al., 2005; Ju et al., 2005; Morozov et al., 1990; Solovyev et al., 2000; Tamai and Meshi, 2001; Zamyatnin et al., 2002, 2004) and, following cargo delivery to PD, the TGBp2 and TGBp3 are proposed to be recycled through the endocytic pathway (Haupt et al., 2005).

The actual form in which the potexvirus RNA is transported between cells remains the subject of debate. Immunodetection of CP associated with fibrillar material embedded within PD, in conjunction with the fact that the CP can move between cells, is consistent with translocation of viral genomic RNA in the form of virions (Oparka et al., 1996; Santa Cruz et al., 1998). However, virion formation per se is not sufficient to promote cell-to-cell movement, as CP mutants that could form virions were unable to move between cells (Forster et al., 1992). In addition, CP mutants with dramatically reduced rates of virion formation were not impacted in terms of the rate of cell-to-cell movement (Lough et al., 2000). Microinjection experiments, utilizing transgenic plants expressing CP, TGBp2, TGBp3, TGBp2 and TGBp3, or TGBp2, TGBp3 plus CP, demonstrated that cell-to-cell movement of the TGBp1–RNA complex, in *Nicotiana benthamiana*, required the presence of TGBp2, TGBp3 and CP (Lough et al., 1998). These results support a model in which viral RNA is trafficked through PD in the form of a TGBp1–CP–RNA complex (Lough et al., 1998, 2000).

In the present study, two potexviruses, *Potato virus X* (PVX) and *White clover mosaic virus* (WClMV), were used to further investigate the molecular determinants for viral cell-to-cell movement. By using an artificial potexvirus defective RNA, encoding a green fluorescent protein (*GFP*) reporter transcriptionally fused to the 5′ and 3′ terminal viral sequences, we identified a *cis*-acting motif that is necessary for recognition and cell-to-cell trafficking of this chimeric RNA. Mapping experiments localized the *cis*-acting element to nucleotides 1–107 of the PVX genome. Our studies also demonstrated that movement of a specific potexvirus requires recognition between the cognate viral RNA, TGBp1, TGBp2, TGBp3 and CP. These findings are discussed with respect to the evolution of processes that control macromolecular trafficking through PD.

Results

Cognate protein–RNA interactions

A series of TGB and CP deletion mutants within the PVX and WClMV genomes were constructed for use in studies aimed at elucidating the specific requirements for cognate protein–viral RNA interaction necessary for cell-to-cell movement (Fig. 1A). Plants of *N. benthamiana* were either mechanically inoculated with infectious viral transcripts, or microprojectile bombarded with plasmids encoding viral sequences, to initiate infections of the PVX and WClMV movement-deficient mutants. Virus/transcript movement was detected by monitoring the level of GFP fluorescence.

In the absence of either TGBp1 or CP, these PVX mutants always remained restricted to the initially infected cells (Fig.

Fig. 1. Specific cognate protein–protein and/or protein–RNA interaction underlies cell-to-cell trafficking of a ribonucleoprotein complex (RNP) in *Nicotiana benthamiana*. (A) Viral constructs based on the potexvirus genome in which *GFP* was inserted and used as a reporter for cell-to-cell transport of RNA. Experiments based on the triple gene block (TGB) and coat protein (CP) open reading frames of *Potato virus X* (PVX) and *White clover mosaic virus* (WClMV). A subset of experiments was performed with a mutant *Tobacco mosaic virus* (TMV) in which the movement protein (MP) was deleted. (B–D) TGBp1 and CP protein or transcripts are translocated between cells with viral RNA. Movement-defective mutant PVX:GFP:ΔCP (or PVX:GFP:ΔTGB1) remained restricted to the initially infected cell (B), whereas when co-bombarded with PVX derived *35S*: :CP (C), or PVX derived *35S*: :TGB1 (D), resulted in fluorescent signals being detected in a number of cells consistent with cell-to-cell movement. (E–G) Transgenic *N. benthamiana* plants expressing WClMV CP (E), TGBp1 (F) or TGBp1, TGBp2, TGBp3 and CP (G) could not rescue PVX:GFP:ΔCP, PVX:GFP:ΔTGB1 or PVX:GFP:ΔTGB1-3ΔCP, respectively. (H) Functional WClMV movement complex (TGBp1, TGBp2, TGBp3 and CP), when expressed in transgenic plants, exhibited cognate RNA specificity in trafficking of WClMV:GFP:ΔTGB1-3ΔCP (compare with G). (I) Absence of any member of the WClMV movement complex (TGBp1, TGBp2, TGBp3 or CP) prevented cell-to-cell transport of the WClMV:GFP:ΔTGB1-3ΔCP. (J) TMV:GFP:ΔMP was not rescued by transgenic plants expressing a functional WClMV movement complex. Inset: TMV MP expressed in *N. benthamiana* rescued the TMV:GFP:ΔMP mutant. Scale bars: B, common to panels C–G, I and J, 100 μm; H, 1000 μm and panel J inset, 400 μm.

1B; Table 1). That both TGBp1 and CP are required for the intercellular movement of infectious RNA was illustrated by co-bombardment experiments in which movement-deficient PVX mutants were rescued by a plasmid encoding the corresponding wild-type gene (Figs. 1C and D; Table 1). As infection was initiated at the single cell level, by transient nuclear expression of constructs that were fused to the *35S* promoter, the extensive cell-to-cell movement of the virus is consistent with co-translocation of TGBp1 and CP, or their transcripts, through PD along with the infectious RNA.

Movement-deficient viral mutants and ectopic expression of viral products were next used to establish the absolute requirement for specific cognate interactions between potexvirus RNA and the viral movement machinery (TGBp1, TGBp2, TGBp3 and CP). Here, PVX:GFP:ΔCP, PVX:GFP:ΔTGB1 and PVX:GFP:ΔTGB1-3ΔCP (Fig. 1A) remained restricted to the initially infected cell, when experiments were performed on transgenic *N. benthamiana* plants expressing WClMV CP (Fig. 1E), WClMV TGBp1 (Fig. 1F) and WClMV TGBp1, TGBp2, TGBp3 and CP (Fig. 1G), respectively (Table 1). The capacity of WClMV TGBp1, TGBp2, TGBp3 and CP expressing plants to rescue cell-to-cell movement of WClMVΔTGB1-3ΔCP, but not the equivalent PVX mutant (compare Figs. 1G and H), demonstrated that the fully assembled and functional WClMV movement complex exhibits cognate RNA specificity (Table 1). Equivalent experiments conducted in the absence of any of these four proteins resulted in restriction of viral infection to the initially infected cells (Fig. 1I; Table 1).

We next addressed the capacity of *Tobacco mosaic virus* (TMV) MP to rescue potexvirus cell-to-cell movement. Control experiments performed with TMV MP transgenic *N. benthamiana* plants confirmed the capacity of TMV MP to rescue cell-to-cell movement of a TMV:GFP:ΔMP mutant (Fig. 1A; Table 1). Parallel experiments performed using transgenic plants expressing WClMV TGBp1, TGBp2, TGBp3 and CP failed to rescue cell-to-cell movement of a TMV:GFP:ΔMP mutant (Fig. 1J; Table 1). Consistent with earlier studies, cell-to-cell movement of *35S*::PVX:GFP:ΔTGB1-3ΔCP and WClMV:GFP:ΔTGB1-3ΔCP was rescued in transgenic plants expressing the TMV MP (Table 1) (Cooper et al., 1996; De Jong and Ahlquist, 1992; Giesman-Cookmeyer et al., 1995; Solovyev et al., 1996). However, cell-to-cell movement of TMV:GFP:ΔMP was not rescued by transgenic plants expressing WClMV TGBp1, TGBp2, TGBp3 and CP (Table 1). These results provide further support for the hypothesis that the potexvirus movement complex exhibits cognate RNA specificity.

Table 1
Proteins and *cis*-acting elements required for cell-to-cell transport of RNA

Plasmid/transcripts used in transient expression assays	Ectopically expressed proteins or co-bombarded plasmids [a]	Infection foci [b] (number of foci per size class, [%])		
		1 cell	2–9 cells	≥10 cells
35S::PVX:GFP	–			1041 (100)
WClMV:GFP	–			673 (100)
35S::PVX:GFP:ΔTGB1	–	353 (100)		
35S::PVX:GFP:ΔCP	–	200 (100)		
35S::PVX:GFP:ΔTGB1	*35S*::PVX TGB1	452 (24)	979 (52)	452 (24)
35S::PVX:GFP:ΔCP	35S::PVX CP	352 (18)	919 (47)	684 (35)
35S::PVX:GFP:ΔCP	*tg* WClMV CP	174 (100)		
35S::PVX:GFP:ΔTGB1	*tg* WClMV TGBp1	631 (100)		
35S::PVX:GFP:ΔTGB1–3ΔCP	*tg* WClMV TGBp1–3 and CP	609 (100)		
WClMV:GFP:ΔTGB1–3ΔCP	–	148 (100)		
WClMV:GFP:ΔTGB1–3ΔCP	*tg* WClMV TGBp1–3 and CP	94 (6)	552 (35)	930 (59)
35S::PVX:GFP:ΔTGB1–3ΔCP	*tg* WClMV TGBp1–3 and CP	609 (100)		
WClMV:GFP:ΔTGB1–3ΔCP	*tg* WClMV TGBp1–3	230 (100)		
WClMV:GFP:ΔTGB1–3ΔCP	*tg* WClMV TGBp2–3 and CP	122 (100)		
TMV:GFP:ΔMP	–	71 (100)		
TMV:GFP:ΔMP	*tg* TMV MP			139 (100)
TMV:GFP:ΔMP	*tg* WClMV TGBp1–3 and CP	215 (100)		
WClMV:GFP:ΔTGB1–3ΔCP	*tg* TMV MP			30 (100)
35S::PVX:GFP:ΔTGB1–3ΔCP	*tg* TMV MP			20 (100)
35S::WClMV	*35S*::WClMV chimeric RNA reporter [c]			458 (100)
35S::WClMV	*35S*::PVX chimeric RNA reporter [d]	0 (0)		
35S::PVX	*35S*::PVX chimeric RNA reporter [e]			1629 (100)
35S::PVX	*35S*::WClMV chimeric RNA reporter [d]	0 (0)		
35S::WClMV	*35S*::GFP	5210 (100)		
35S::PVX	*35S*::GFP	1851 (100)		
–	*35S*::GFP	4924 (100)		

[a] Proteins ectopically expressed in transgenic *Nicotiana benthamiana* plants (*tg*) or by co-bombardment of *35S*-driven plasmids.
[b] Number of infection foci and percentage of total infection foci in each size class recorded 4–7 days post-bombardment.
[c] WClMV chimeric RNA reporter: *GFP* ORF was inserted, in-frame, into the WClMV genome flanked by the 871 5′- and 155 3′-terminal nucleotides.
[d] Chimeric RNA reporter co-bombarded (n = 4–6 leaves) with the heterologous virus failed to reveal fluorescence.
[e] PVX chimeric RNA reporter: *GFP* ORF was inserted, in-frame, into the PVX genome flanked by the 548 5′- and 370 3′-terminal nucleotides.

A cis-acting element is required for cell-to-cell movement of potexvirus RNA

To test the hypothesis that *cis*-acting sequences, located in the 5′ or 3′ region of the potexviral genome, are necessary for its cell-to-cell movement, two chimeric RNA reporters were constructed based on the PVX and WClMV genomes. These chimeric RNA reporters were driven by the *35S* promoter and contained the green fluorescent protein (*GFP*) ORF inserted, in-frame, into the PVX and WClMV genome such that it was flanked by 5′ and 3′ viral sequences (Fig. 2A).

Analysis of GFP fluorescence was used to assay for the action of these elements within the viral genome. Introduction of each chimeric RNA reporter into plant tissues gave rise to a fluorescent signal that was always confined to the initially bombarded cells (Fig. 2B; Table 2). In contrast, co-

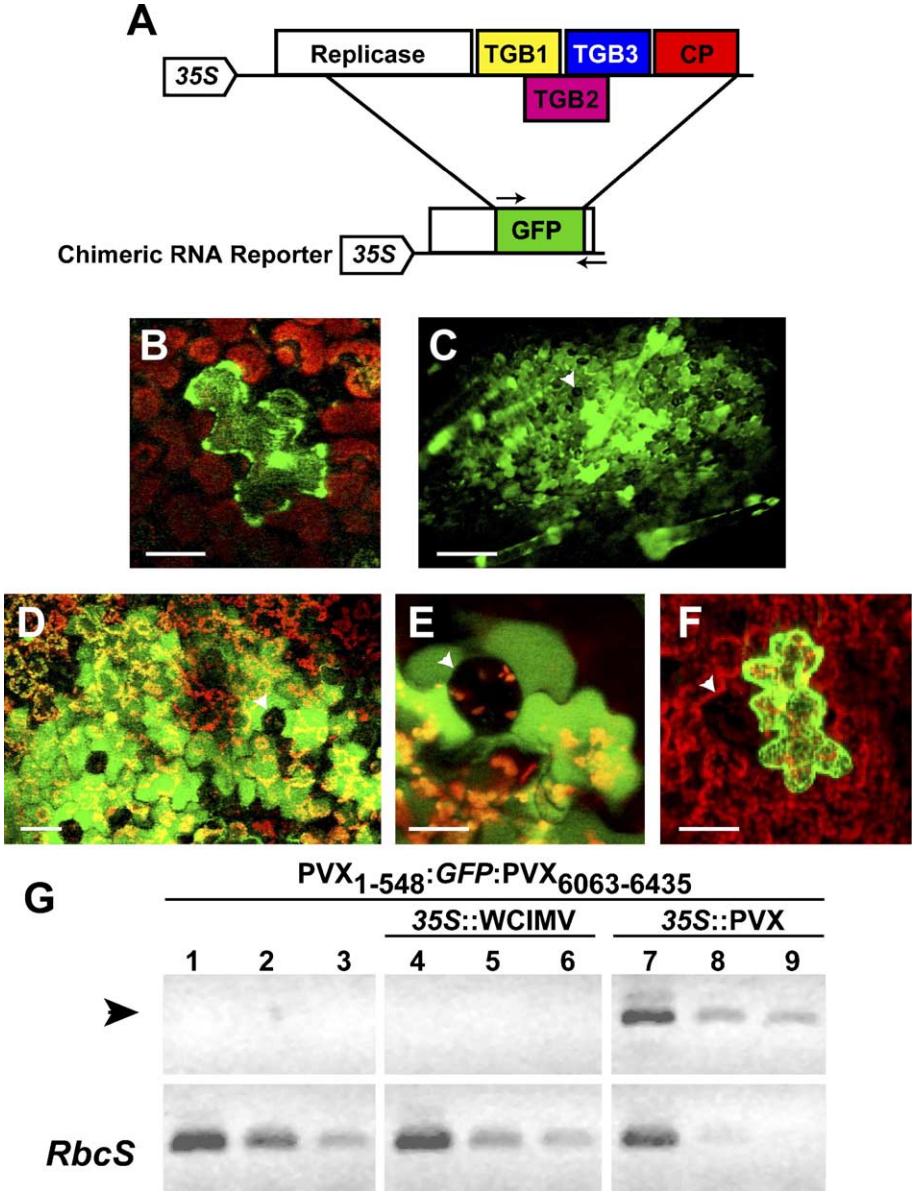


Fig. 2. Cell-to-cell trafficking of chimeric RNA reporter potentiated by potexvirus *cis*-acting sequence elements. (A) Chimeric RNA reporter developed using 5′ and 3′ sequences derived from PVX or WClMV. Specificity of *cis*-acting elements was investigated using co-bombardment with *35S*: :PVX or *35S*: :WClMV. (B) Expression of the chimeric $PVX_{1–548}$:*GFP*:$PVX_{6065–6435}$ reporter was restricted to the initially bombarded cells. Equivalent results were obtained with the chimeric $WClMV_{1–872}$:*GFP*:$WClMV_{5692–5846}$ reporter. (C) Extensive cell-to-cell movement of the $WClMV_{1–872}$:*GFP*:$WClMV_{5692–5846}$ reporter following co-bombardment with *35S*: :WClMV. (D and E) Extensive cell-to-cell movement of the chimeric $PVX_{1–548}$:*GFP*:$PVX_{6065–6435}$ reporter following co-bombardment with *35S*: :PVX. (C–E) Both chimeric RNA reporter systems were excluded from symplasmically isolated guard cells (dart). (F) Control experiments in which a *35S*: :*GFP* reporter, lacking *cis*-acting elements, was co-bombarded with *35S*: :PVX (equivalent results were obtained with *35S*: :WClMV). (G) RT-PCR analysis of extracted RNA confirmed that movement and expression of the chimeric $PVX_{1–548}$:*GFP*:$PVX_{6065–6435}$ reporter require cognate protein recognition. Serial dilutions of RNA (1:1 [lanes 1, 4, 7], 1:5 [lanes 2, 5, 8], 1:25 [lanes 3, 6, 9]). Internal loading control based on analysis of *RbcS*. Position of $PVX_{1–548}$:*GFP*:$PVX_{6065–6435}$ specific primers indicated in panel A (arrows). Scale bars: B, E and F, 100 μm; C, 800 μm; D, 250 μm.

Table 2
Localization of minimal sequence required for cell-to-cell transport of chimeric GFP RNA reporter

Plasmids used in transient expression assays	Co-bombarded plasmid	Infection foci[a] (number of foci per size class, [%])	
		1 cell	≥10 cells
–	*35S*: :$PVX_{1–336}$:*GFP*:$PVX_{6065–6435}$	4 (100)	
–	*35S*: :$PVX_{1–182}$:*GFP*:$PVX_{6065–6435}$	5 (100)	
–	*35S*: :$PVX_{1–143}$:*GFP*:$PVX_{6065–6435}$	18 (100)	
–	*35S*: :$PVX_{1–107}$:*GFP*:$PVX_{6065–6435}$	6 (100)	
–	*35S*: :$PVX_{1–88}$:*GFP*:$PVX_{6065–6435}$	1 (100)	
–	*35S*: :$PVX_{1–49}$:*GFP*:$PVX_{6065–6435}$	4 (100)	
35S: :WClMV	*35S*: :$PVX_{1–336}$:*GFP*:$PVX_{6065–6435}$	3 (100)	
35S: :WClMV	*35S*: :$PVX_{1–182}$:*GFP*:$PVX_{6065–6435}$	5 (100)	
35S: :WClMV	*35S*: :$PVX_{1–143}$:*GFP*:$PVX_{6065–6435}$	82 (100)	
35S: :WClMV	*35S*: :$PVX_{1–107}$:*GFP*:$PVX_{6065–6435}$	443 (100)	
35S: :WClMV	*35S*: :$PVX_{1–88}$:*GFP*:$PVX_{6065–6435}$	664 (100)	
35S: :WClMV	*35S*: :$PVX_{1–49}$:*GFP*:$PVX_{6065–6435}$	580 (100)	
35S: :PVX	*35S*: :$PVX_{1–336}$:*GFP*:$PVX_{6065–6435}$		1260 (100)
35S: :PVX	*35S*: :$PVX_{1–182}$:*GFP*:$PVX_{6065–6435}$	10 (2)	576 (98)
35S: :PVX	*35S*: :$PVX_{1–143}$:*GFP*:$PVX_{6065–6435}$	203 (36)	359 (64)
35S: :PVX	*35S*: :$PVX_{1–107}$:*GFP*:$PVX_{6065–6435}$	250 (56)	170 (38)
35S: :PVX	*35S*: :$PVX_{1–88}$:*GFP*:$PVX_{6065–6435}$	510 (100)	
35S: :PVX	*35S*: :$PVX_{1–49}$:*GFP*:$PVX_{6065–6435}$	93 (100)	

[a] Number of infection foci and percentage of total infection foci in each size class recorded 4–7 days post-bombardment. Data combined from 4 independent experiments.

bombardment with the cognate potexvirus, but not the heterologous potexvirus, led to the detection of fluorescent signal in foci containing many epidermal cells (Figs. 2C and D; Table 1), consistent with cell-to-cell movement and expression of the chimeric RNA reporter. Extraction of RNA from co-bombarded leaves and subsequent RT-PCR analyses confirmed that evidence of chimeric RNA accumulation only occurred when co-bombarded with the cognate, and not the heterologous, potexvirus (data not presented). Evidence consistent with movement through PD was provided by the absence of fluorescent signal in guard cells (Figs. 2C, D and E) which lack functional PD in mature leaves. As expected, a *GFP* reporter lacking viral *cis*-acting sequences was always confined to the initially bombarded cells (Fig. 2F; Table 1).

Specificity of the *cis*-acting elements, in mediating the cell-to-cell movement of the chimeric RNA reporter, was confirmed by co-bombardment experiments conducted using heterologous potexviruses. In these experiments, the PVX-derived chimeric RNA reporter was co-bombarded with either *35S*: :WClMV or *35S*: :PVX. Only cognate virus-chimeric RNA reporter combinations (i.e., co-bombardment of PVX-derived chimeric RNA reporter with *35S*: :PVX but not *35S*: :WClMV, and *vice versa*), resulted in the development of detectable fluorescent foci (Table 1). In addition, RT-PCR analysis of RNA extracted from these experimental tissues provided further support for the hypothesis that *cis*-acting elements mediated the cell-to-cell trafficking of the chimeric RNA reporter. Here, amplification of the chimeric RNA reporter was restricted to treatments in which the reporter was present with its cognate potexvirus (Fig. 2G).

Mapping the cis-acting element

To identify the location of the *cis*-acting element, a series of chimeric RNA reporters were constructed in which the 5′ PVX sequences were reduced from 548 to 49 (Fig. 3A). Mapping experiments performed with these constructs identified the location of the *cis*-acting element to be within nucleotides 1–107 of the PVX genome (Fig. 3A; Table 2). RT-PCR analysis was used to confirm that only cognate virus-chimeric RNA reporter combinations resulted in RNA amplification within detected fluorescent foci (data not presented).

In tissues co-bombarded with *35S*: :PVX and *35S*: :PVX_{182}:*GFP*, *35S*: :PVX_{143}:*GFP* or *35S*: :PVX_{107}:*GFP*, we observed a bimodal pattern in which fluorescence was detected in greater than 10 cells, or it was restricted to the initially bombarded cells (Table 2). The situation in which fluorescent signal was associated with single cells probably reflected transient expression derived from the chimeric RNA reporter alone, or replication of reporter variants that were unable to move through PD.

Conserved RNA secondary structures, required for efficient replication, have been defined within PVX nucleotides 1–230 (Kwon et al., 2005; Miller et al., 1998). Interestingly, although of lower overall efficiency, the *35S*: :PVX_{107}:*GFP* reporter retained the capacity for cell-to-cell movement. This supports the hypothesis that a structural element required for replication (Miller et al., 1998), within nucleotides 1–107 (Fig. 3B), also acts as a *cis*-acting element essential for cell-to-cell movement of the chimeric RNA reporter.

Discussion

In the present study, we provide evidence in support of the hypothesis that specific *cis*-acting elements mediate the selective recognition and cell-to-cell transport of potexvirus RNA. This model was founded on our observation that ectopic expression of a functional WClMV movement complex, comprised of TGBp1, TGBp2, TGBp3 and CP, could rescue movement-deficient WClMV but not the equivalent PVX mutants (Fig. 1; Table 1). The chimeric RNA reporter system developed to probe the molecular basis for this specificity represents an artificial, defective, potexvirus RNA encoding a *GFP* reporter transcriptionally fused to the 5′ and 3′ terminal viral sequences. The *cis*-acting element, located at the 5′ region of the viral RNA, was shown to be necessary for intercellular transport of the chimeric RNA reporter (Fig. 2; Table 2).

Mutational analysis of predicted and characterized RNA secondary structural elements, located within the 5′ region of the PVX genome, indicated that nucleotides 1–107 are required for viral plus-strand accumulation (Kwon et al., 2005; Miller et al., 1998). In view of the involvement of 5′ sequences in both replication and movement (Atabekov et al., 2001; Buck, 1996), it is possible that replication of genomic RNA is coordinated with the cell-to-cell movement of viral RNA (Fig. 3A). Furthermore, our studies now establish that cognate recognition, most likely involving a secondary structural element

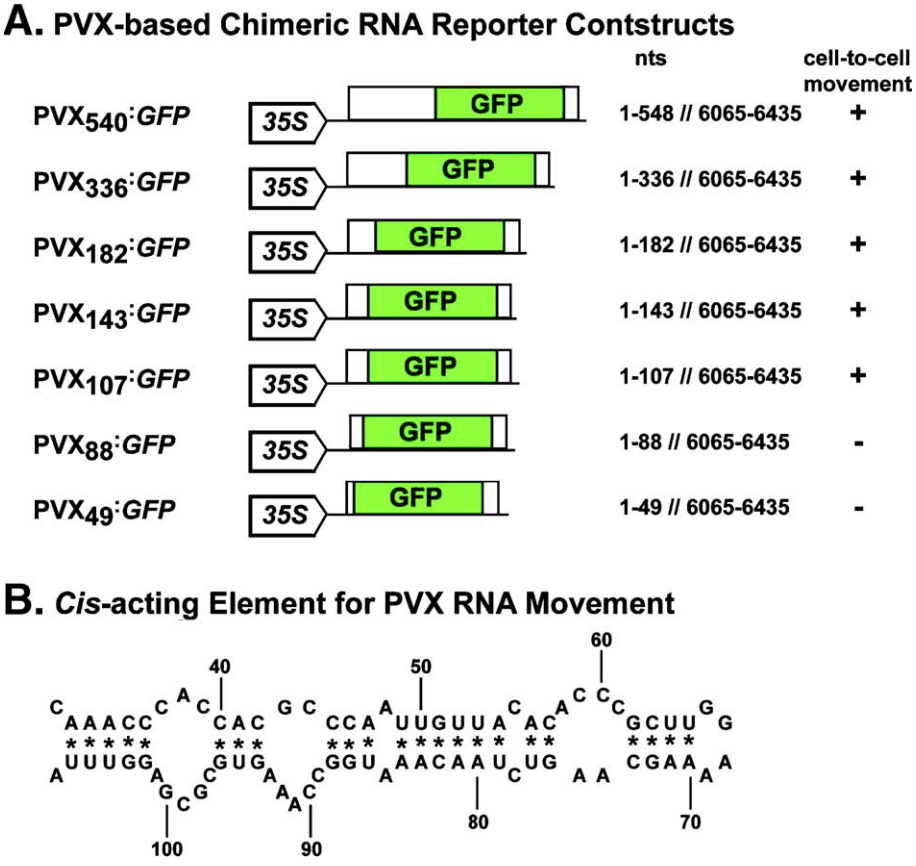


Fig. 3. Localization of the minimal *cis*-acting sequence essential for cell-to-cell trafficking of the chimeric RNA reporter. (A) Chimeric RNA reporter constructs were developed using 548, 336, 182, 143, 107, 88 or 49 5′ viral nucleotides. Extensive cell-to-cell movement (>50 cells) of PVX_{336}:*GFP*, PVX_{182}:*GFP*, PVX_{143}:*GFP* or PVX_{107}:*GFP* resulted following co-bombardment with *35S*: :PVX. (B) A conserved element of RNA secondary structure located within nucleotides 1–107, previously implicated in replication (Miller et al., 1998), likely functions as the *cis*-acting element essential for cognate virus-chimeric RNA reporter recognition.

within the PVX nucleotides 1–107 (Fig. 3B), is required for potexvirus RNA movement.

Potexviruses may move through PD as a virus particle (Atabekov et al., 2000, 2001; Oparka et al., 1996; Santa Cruz et al., 1998) or as a viral RNA–CP–TGBp1–complex (Lough et al., 1998, 2000). While our present findings are consistent with the viral ribonucleoprotein complex hypothesis, they do not formally exclude the possibility that potexvirus RNA moves cell-to-cell in the form of a virion. However, we favor the model that the cell-to-cell transport of potexvirus RNA may involve the following steps: (a) recognition of the 5′ *cis*-acting element(s) by the CP, (b) subsequent recruitment of TGBp1, and (c) interaction of this CP–TGBp1–RNA complex with TGBp2 and TGBp3 containing vesicles that mediate delivery to the cell periphery for docking at the PD orifice.

The basis for cognate recognition of potexvirus RNA by coat protein is most likely based on the sequence and adopted tertiary structure of the CP. Cognate recognition is achieved despite relatively high levels of homology shared by WClMV and PVX CP (48%). In the absence of recognition, the TGB products lack a capacity to traffic the heterologous RNA despite similarity between the products in the range of 40 to 45%. A role for the potexvirus CP in cognate recognition of sequences located at the extreme 5′ end of the potexvirus genome is supported by in vitro initiation of *Papaya mosaic virus* virion assembly (Sit et al., 1994). In addition, secondary structures are known to be involved in CP binding to PVX genomic RNA (Kwon et al., 2005; Miller et al., 1998). A similar finding has been reported for a number of other plus-strand RNA viruses as well as for retroviruses (Clever et al., 1995; De Guzman et al., 1998; Fosmire et al., 1992; Fujimura et al., 1990; Houwing and Jaspars, 1982; Satyanarayana et al., 2004; Turner et al., 1988; Wei et al., 1992; Zhong et al., 1992).

Naturally occurring potexvirus defective RNAs, similar to the chimeric RNA reporter described in the present study, have been described based on *Clover yellow mosaic virus*, *Bamboo mosaic virus* and *Cassava common mosaic virus* (Calvert et al., 1996; White et al., 1991; Yeh et al., 1999). These so-called defective RNAs are identical to the parent virus from which they are derived over the entire length of the defective RNA. Consequently, the defective RNA will also likely be trafficked cell to cell through cognate recognition by the movement machinery of the parent virus.

The *Bamboo mosaic virus* (BaMV) satellite RNA (satRNA) is similar in nature to our chimeric RNA reporter, in that it has only limited homology with its associated helper potexvirus, BaMV (Annamalai et al., 2003; Lin and Hsu, 1994). Based on nucleotide identity (65%) and similarity in predicted RNA secondary structure, within the first 90 nucleotides of their genomes, it seems likely that the satRNA has retained a *cis*-

acting element that functions in cognate recognition by the BaMV movement machinery (N-S. Lin, personal communication). Finally, helper virus-assisted movement of completely non-homologous satellite RNA (Scholthof et al., 1999) suggests that, in many instances, recognition may well be mediated by structurally conserved elements as opposed to homologous primary sequences.

It is generally accepted that the majority of plant viral MPs function in cell-to-cell movement of the infectious nucleic acids in the absence of a requirement for cognate viral sequence recognition (Heinlein and Epel, 2004; Lucas, 2006; Waigmann et al., 2004). This assertion was founded on experiments including the functional rescue of RNA movement when the MP was expressed, in *cis*, in the context of a hybrid viral genome (Cooper et al., 1996; De Jong and Ahlquist, 1992; Giesman-Cookmeyer et al., 1995; Solovyev et al., 1996), or in *trans*, using transgenic plants or co-bombardment experiments (Giesman-Cookmeyer et al., 1995; Lauber et al., 1998; Morozov et al., 1997). However, the possibility cannot be discounted that certain cellular boundaries, within the host plant, may require some degree of additional cognate protein–RNA specificity to permit cell-to-cell transport of RNA. Indeed, such a requirement may explain why for some viruses, including TMV and *Red clover necrotic mosaic virus*, entry into the plant vascular system requires both the MP and CP (Ding et al., 1996; Saito et al., 1990; Vaewhongs and Lommel, 1995).

Materials and methods

Viral constructs

Constructs based on PVX including *35S*::PVX:GFP, *35S*::TGB1 and *35S*::CP were as previously described (Lough et al., 2000; Santa Cruz et al., 1996). Infectious constructs for PVX:GFP:ΔTGB1-3ΔCP (Voinnet et al., 2000) and TMV:GFP:ΔMP (Shivprasad et al., 1999) were the generous gift of Simon Santa Cruz. The clone WClMV:GFP:ΔTGB1-3ΔCP was based on WClMV:GFP (Lough et al., 2000). Chimeric RNA reporter constructs were derived from genomic sequences of either PVX or WClMV. A *GFP* reporter (mGFP5; Haseloff et al., 1997) was inserted, in-frame, into the PVX genome at *Bam*HI–*Hpa*I (548–6065) and the extreme 3′ 370 nucleotides (6065–6435) were fused to the *GFP* ORF. A further series of chimeric RNA reporter constructs incorporating 336, 182, 143, 107, 88 or 49 5′ nucleotides (*35S*::PVX_{1-336}:*GFP*:$PVX_{6065-6435}$, *35S*::PVX_{1-182}:*GFP*:$PVX_{6065-6435}$, *35S*::PVX_{1-143}:*GFP*:$PVX_{6065-6435}$, *35S*::PVX_{1-107}:*GFP*:$PVX_{6065-6435}$, *35S*::$_{PVX1-88}$:*GFP*:$PVX_{6065-6435}$, *35S*::PVX_{1-49}:*GFP*:$PVX_{6065-6435}$) was made by PCR using PVX_{1-548}:*GFP*:$PVX_{6065-6435}$ as the PCR template. An equivalent chimeric RNA reporter construct was produced using WClMV sequences with *GFP* inserted inframe at *Sac*II–*Nru*I (872–5691) and the extreme 3′ 155 nucleotides (5692–5846) were fused to the *GFP* ORF. All chimeric RNA reporter constructs were confirmed by sequence analysis. The nucleotide numbering of viral sequences was as previously described for PVX (Huisman et al., 1988) and WClMV (Beck et al., 1990). All chimeric RNA reporter constructs were fused to the *35S* promoter such that the first transcribed base was identical to that of the corresponding virus. The infectious clone *35S*::PVX was engineered by deletion of *GFP* (*Eag*I–*Bst*BI) from *35S*::PVX:GFP (Santa Cruz et al., 1996). The *35S* promoter was fused to the WClMV genome by insertion at the *Stu*I site of pCass2 (Shi et al., 1997).

Transgenic plants

WClMV TGB and CP coding sequences were subcloned into the binary vector pART27 (Gleave, 1992) and introduced into *N. benthamiana* by *Agrobacterium*-mediated transformation (Horsch et al., 1985) as described previously (Lough et al., 1998). Similarly, plants expressing TGB1–3 or TGB1–3 plus CP were generated as described previously (Lough et al., 2000, 2001). Transgenic plants expressing the *Tobacco mosaic virus* movement protein were the generous gift of Mike Deom (University of Georgia, Athens, GA).

Microprojectile bombardment and fluorescence analysis

Non-linearized infectious plasmids were introduced into epidermal cells on the adaxial surface of mature *N. benthamiana* leaves by microprojectile bombardment as described (Lough et al., 1998, 2000). Transgenic plants expressing WClMV products were as described (Lough et al., 1998, 2000). Analysis of the spatial distribution of GFP in plant tissues was performed 4–7 days post-bombardment (unless otherwise stated), using either confocal laser scanning microscopy (Leica model TCS 4D) or epifluorescence microscopy (Leica MZFLIII stereomicroscope equipped with a DC200 digital camera).

RT-PCR analysis

Poly(A)$^+$ mRNA was extracted from plant tissues, following microprojectile bombardment, using oligo(dT) magnetic beads (Dynal). One step RT-PCR (Gibco-BRL) reactions were performed on 5-fold serial dilutions of RNA. Specific reactions for the PVX-based chimeric RNA reporter were performed in a 20 μl reaction volume using 5 pmol of each primer (5′-ATCTAGCAGGATCCCAGTAAAGGAGAAGAACTTTT-3′ and 5′-TAGGCGTCGGTTATGTAGACGTAGT-3′). *RbcS* was used as an internal control with reactions as performed above using 5 pmol of each primer (5′-AATTGCTCCTGGCT-CAAATC-3′ and 5′-GCTTCCTCAGTTCTTTCCTC-3′). RT-PCR was performed using: 1 cycle of 50 °C for 30 min, 94 °C for 2 min; 30 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 1 min; followed by 1 cycle of 72 °C for 5 min.

Acknowledgments

We thank Simon Santa Cruz (SCRI, Invergowrie, U.K.) for PVX and TMV infectious constructs, Cynthia Hemenway for critical reading of the manuscript, Natalie Netzler for technical

assistance, Vaughan Cruden and Cameron Redfearn for assistance with plant propagation and Samantha Barling-Silva for assistance with image production. This work was supported by grants from the New Zealand Foundation for Research Science and Technology (C06X0001), the Department of Energy, Division of Energy Biosciences (DE-FG03-94ER20134) and National Science Foundation (IBN-04-44725).

References

Annamalai, P., Hsu, Y.-H., Liu, Y.P., Tsai, C.H., Lin, N.-S., 2003. Structural and mutational analyses of *cis*-acting sequences in the 5′-untranslated region of satellite RNA of Bamboo Mosaic Potexvirus. Virology 311, 229–239.

Aoki, K., Kragler, F., Xoconostle-Cazares, B., Lucas, W.J., 2002. A subclass of plant heat shock cognate 70 chaperones carries a motif that facilitates trafficking through plasmodesmata. Proc. Natl. Acad. Sci. U.S.A. 99, 16342–16347.

Atabekov, J.G., Rodionova, N.P., Karpova, O.V., Kozlovsky, S.V., Poljakov, V.Y., 2000. The movement protein-triggered in situ conversion of Potato Virus X virion RNA from a nontranslatable into a translatable form. Virology 271, 259–263.

Atabekov, J.G., Rodionova, N.P., Karpova, O.V., Kozlovsky, S.V., Novikov, V.K., Arkhipenko, M.V., 2001. Translational activation of encapsidated potato virus X RNA by coat protein phosphorylation. Virology 286, 466–474.

Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Bassell, G.J., Kelic, S., 2004. Binding proteins for mRNA localization and local translation, and their dysfunction in genetic neurological disease. Curr. Opin. Neurobiol. 14, 574–581.

Bassell, G.J., Twiss, J.L., 2006. RNA exodus to Israel: RNA controlling function in the far reaches of the neuron. EMBO Rep. 7, 31–35.

Baulcombe, D.C., Chapman, S., Santa Cruz, S., 1995. Jellyfish green fluorescent protein as a reporter for virus infections. Plant J. 7, 1045–1053.

Beck, D.L., Forster, R.L.S., Bevan, M.W., Boxen, K.A., Lowe, S.C., 1990. Infectious transcripts and nucleotide sequence of cloned cDNA of the potexvirus white clover mosaic virus. Virology 177, 152–158.

Beck, D.L., Guilford, P.J., Voot, D.M., Andersen, M.T., Forster, R.L.S., 1991. Triple gene block proteins of white clover mosaic potexvirus are required for transport. Virology 183 (2), 695–702.

Buck, K.W., 1996. Comparison of the replication of positive-stranded RNA viruses of plants and animals. Adv. Virus Res. 47, 159–251.

Calvert, L.A., Cuervo, M.I., Ospina, M.D., Fauquet, C.M., Ramirez, B.-C., 1996. Characterization of cassava common mosaic virus and a defective RNA species. J. Gen. Virol. 77, 525–530.

Chapman, S., Hills, G., Watts, J., Baulcombe, D., 1992. Mutational analysis of the coat protein gene of potato virus X effects on virion morphology and viral pathogenicity. Virology 191, 223–230.

Choi, S.-B., Wang, C., Muench, D.G., Ozawa, K., Franceschi, V.R., Wu, Y., Okita, T.W., 2000. Messenger RNA targeting of rice seed storage proteins to specific ER subdomains. Nature 407, 765–767.

Clever, J., Sassetti, C., Parslow, T.G., 1995. RNA secondary structure and binding sites for gag gene products in the 5′ packaging signal of human immunodeficiency virus type 1. J. Virol. 69, 2101–2109.

Cooper, B., Schmitz, I., Rao, A.L.N., Beachy, R.N., Dodds, J.A., 1996. Cell-to-cell transport of movement-defective cucumber mosaic and tobacco mosaic viruses in transgenic plants expressing heterologous movement protein genes. Virology 216, 208–213.

De Guzman, R.N., Wu, Z.R., Stalling, C.C., Pappalardo, L., Borer, P.N., Summers, M.F., 1998. Structure of the HIV-1 nucleocapsid protein bound to the SL3 psi-RNA recognition element. Science 279, 384–388.

De Jong, W., Ahlquist, P., 1992. A hybrid plant RNA virus made by transferring the noncapsid movement protein from a rod-shaped to an icosahedral virus is competent for systemic infection. Proc. Natl. Acad. Sci. U.S.A. 89, 6808–6812.

Ding, X., Shintaku, M.H., Carter, S.A., Nelson, R.S., 1996. Invasion of minor veins of tobacco leaves inoculated with tobacco mosaic virus mutants defective in phloem-dependent movement. Proc. Natl. Acad. Sci. U.S.A. 93, 11155–11160.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.

Forster, R.L.S., Beck, D.L., Guilford, P.J., Voot, D.M., van Dolleweerd, C.J., Andersen, M.T., 1992. The coat protein of white clover mosaic potexvirus has a role in facilitating cell-to-cell transport in plants. Virology 191, 480–484.

Fosmire, J.A., Hwang, K., Makino, S., 1992. Identification and characterization of a coronavirus packaging signal. J. Virol. 66, 3522–3530.

Fujimura, T., Esteban, R., Esteban, L.M., Wickner, L.B., 1990. Portable encapsidation signal of the L-A double-stranded RNA virus of *S. cerevisiae*. Cell 62, 819–828.

Giesman-Cookmeyer, D., Silver, S., Vaewhongs, A.A., Lommel, S.A., Deom, C.M., 1995. Tobamovirus and dianthovirus movement proteins are functionally homologous. Virology 213, 38–45.

Gleave, A.P., 1992. A versatile binary vector system with T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol. Biol. 20, 1203–1207.

Haseloff, J., Siemering, K.R., Prasher, D.C., Hodge, S., 1997. Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proc. Natl. Acad. Sci. U.S.A. 94, 2122–2127.

Haupt, S., Cowan, G.H., Ziegler, A., Roberts, A.G., Oparka, K.J., Torrance, L., 2005. Two plant-viral movement proteins traffic in the endocytic recycling pathway. Plant Cell 17, 164–181.

Haywood, V., Yu, T.S., Huang, N.C., Lucas, W.J., 2005. Phloem long-distance trafficking of *GIBBERELLIC ACID-INSENSITIVE* RNA regulates leaf development. Plant J. 42, 49–68.

Heinlein, M., Epel, B.L., 2004. Macromolecular transport and signaling through plasmodesmata. Int. Rev. Cytol. 235, 93–164.

Horsch, R.B., Fry, J.E., Hoffman, N.L., Eichholz, D., Rogers, S.G., Fraley, R.T., 1985. A simple and general method for transferring genes into plants. Science 227, 1229–1231.

Houwing, C., Jaspars, E., 1982. Protein binding sites in nucleation complexes of alfalfa mosaic virus RNA 4. Biochemistry 21, 3408–3414.

Huang, T., Bohlenius, H., Eriksson, S., Parcy, F., Nilsson, O., 2005. The mRNA of the *Arabidopsis* gene *FT* moves from leaf to shoot apex and induces flowering. Science 309, 1694–1696.

Huisman, M.J., Linthorst, H.J.M., Bol, J.F., Cornelissen, B.J.C., 1988. The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. J. Gen. Virol. 69, 1789–1798.

Ju, H.J., Samuels, T.D., Wang, Y.S., Blancaflor, E., Payton, M., Mitra, R., Krishnamurthy, K., Nelson, R.S., Verchot-Lubicz, J., 2005. The potato virus X TGBp2 movement protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol. 138, 1877–1895.

Kim, M., Canio, W., Kessler, S., Sinha, N., 2001. Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. Science 293, 287–289.

Kim, J.Y., Rim, Y., Wang, J., Jackson, D., 2005. A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. Genes Dev. 19, 788–793.

Kwon, S.J., Park, M.R., Kim, K.W., Plante, C.A., Hemenway, C.L., Kim, K.H., 2005. cis-Acting sequences required for coat protein binding and in vitro assembly of Potato Virus X. Virology 334, 83–97.

Lauber, E., Bleykasten-Grosshans, C., Erhardt, M., Bouzoubaa, S., Jonard, G., Richards, K.E., Guilley, H., 1998. Cell-to-cell movement of beet necrotic yellow vein virus: I. Heterologous complementation experiments provide evidence for specific interactions among the triple gene block proteins. Mol. Plant Microbe Interact. 11, 618–625.

Lazarowitz, S.G., Beachy, R.N., 1999. Viral movement proteins as probes for intracellular and intercellular trafficking in plants. Plant Cell 11, 535–548.

Lin, N.-S., Hsu, Y.-H., 1994. A satellite RNA associated with bamboo mosaic potexvirus. Virology 202, 707–714.

Lindbo, J.A., Dougherty, W.G., 2005. Plant pathology and RNAi: a brief history. Annu. Rev. Phytopathol. 43, 191–204.

Lough, T.J., Lucas, W.J., 2006. Integrative plant biology: role of phloem long-distance macromolecular trafficking. Annu. Rev. Plant Biol. 57, 203–232.

Lough, T.J., Shash, K., Xoconostle-Cazares, B., Hofstra, K.R., Beck, D.L., Balmori, E., Forster, R.L.S., Lucas, W.J., 1998. Molecular dissection of the mechanism by which potexvirus triple gene block proteins mediate cell-to-cell transport of infectious RNA. Mol. Plant-Microbe Interact. 11, 801–814.

Lough, T.J., Netzler, N.E., Emerson, S.J., Sutherland, P., Carr, F., Beck, D.L., Lucas, W.J., Forster, R.L.S., 2000. Cell-to-cell movement of potexviruses: evidence for a ribonucleoprotein complex involving the coat protein and first triple gene block protein. Mol. Plant-Microbe Interact. 13, 674–962.

Lough, T.J., Emerson, S.J., Lucas, W.J., Forster, R.L., 2001. Trans-complementation of long-distance movement of White clover mosaic virus triple gene block (TGB) mutants: phloem-associated movement of TGBp1. Virology 288, 18–28.

Lucas, W.J., 2006. Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. Virology 344, 169–184.

Meins Jr., F., Si-Ammour, A., Blevins, T., 2005. RNA silencing systems and their relevance to plant development. Annu. Rev. Cell Dev. Biol. 21, 297–318.

Miki, T., Takano, K., Yoneda, Y., 2005. The role of mammalian Staufen on mRNA traffic: a view from its nucleocytoplasmic shuttling function. Cell Struct. Funct. 30, 51–56.

Miller, E.D., Plante, C.A., Kim, K.H., Brown, J.W., Hemenway, C., 1998. Stem–loop structure in the 5′ region of Potato Virus X genome required for plus-strand RNA accumulation. J. Mol. Biol. 284, 591–608.

Mlotshwa, S., Voinnet, O., Mette, M.F., Matzke, M., Vaucheret, H., Ding, S.W., Pruss, G., Vance, V.B., 2002. RNA silencing and the mobile silencing signal. Plant Cell 14, S289–S301.

Morozov, S.Y., Solovyev, A.G., 2003. Triple gene block: modular design of a multifunctional machine for plant virus movement. J. Gen. Virol. 84, 1351–1366.

Morozov, S.Y., Miroshnichenko, N.A., Zelenina, D.A., Fedorkin, O.N., Solovijev, A.G., Lukasheva, L.I., Atabekov, J.C., 1990. Expression of RNA transcripts of potato virus X full-length and subgenomic cDNAs. Biochimie 72, 677–684.

Morozov, S.Y., Fedorkin, O.N., Juttner, G., Schiemann, J., Baulcombe, D.C., Atabekov, J.G., 1997. Complementation of a potato virus X mutant mediated by bombardment of plant tissues with cloned viral movement protein genes. J. Gen. Virol. 78, 2077–2083.

Napoli, C., Lemieux, C., Jorgensen, R., 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous gene in trans. Plant Cell 2, 279–289.

Oparka, K.J., 2004. Getting the message across: how do plant cells exchange macromolecular complexes? Trends Plant Sci. 9, 33–41.

Oparka, K.J., Roberts, A.G., Roberts, I.M., Prior, D.A.M., Santa Cruz, S., 1996. Viral coat protein is targeted to, but does not gate, plasmodesmata during cell-to-cell movement of potato virus X. Plant J. 10, 805–813.

Qi, Y., Pelissier, T., Itaya, A., Hunt, E., Wassenegger, M., Ding, B., 2004. Direct role of a viroid RNA motif in mediating directional RNA trafficking across a specific cellular boundary. Plant Cell 16, 1741–1752.

Ruiz-Medrano, R., Xoconostle-Cázares, B., Lucas, W.J., 1999. Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. Development 126, 4405–4419.

Saito, T., Yamanaka, K., Okada, Y., 1990. Long-distance movement and viral assembly of tobacco mosaic virus mutants. Virology 176, 329–336.

Santa Cruz, S., Chapman, S., Roberts, A.G., Roberts, I.M., Prior, D.A.M., Oparka, K.J., 1996. Assembly and movement of a plant virus carrying a green fluorescent protein overcoat. Proc. Natl. Acad. Sci. U.S.A. 93, 6286–6290.

Santa Cruz, S., Roberts, A.G., Prior, D.A.M., Chapman, S., Oparka, K.J., 1998. Cell-to-cell and phloem-mediated transport of potato virus X. The role of virions. Plant Cell 10, 495–510.

Satyanarayana, T., Gowda, S., Ayllon, M.A., Dawson, W.O., 2004. Closterovirus bipolar virion: evidence for initiation of assembly by minor coat protein and its restriction to the genomic RNA 5′ region. Proc. Natl. Acad. Sci. U.S.A. 101, 799–804.

Scholthof, K.B., Jones, R.W., Jackson, A.O., 1999. Biology and structure of plant satellite viruses activated by icosahedral helper viruses. Top. Microbiol. Immunol. 239, 123–143.

Shi, B.-J., Ding, S.-W., Symons, R.H., 1997. Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus. J. Gen. Virol. 78, 1181–1185.

Shivprasad, S., Pogue, G.P., Lewandowski, D.J., Hidalgo, J., Donson, J., Grill, L.K., Dawson, W.O., 1999. Heterologous sequences greatly affect foreign gene expression in tobacco mosaic virus-based vectors. Virology 255, 312–323.

Sit, T.L., Leclerc, D., AbouHaidar, M.G., 1994. The minimal 5′ sequence for in vitro initiation of papaya mosaic potexvirus assembly. Virology 199, 238–242.

Solovyev, A.G., Zelenina, D.A., Savenkov, E.I., Grdzelishvili, V.Z., Morozov, S.Y., Lesemann, D.-E., Maiss, E., Casper, R., Atabekov, J.G., 1996. Movement of a Barley Stripe Mosaic Virus chimera with a tobacco mosaic virus movement protein. Virology 217, 435–441.

Solovyev, A.G., Stroganova, T.A., Zamyatnin Jr., A.A., Fedorkin, O.N., Schiemann, J., Morozov, S.Y., 2000. Subcellular sorting of small membrane-associated triple gene block proteins: TGBp3-assisted targeting of TGBp2. Virology 269, 113–127.

Tamai, A., Meshi, T., 2001. Cell-to-cell movement of Potato Virus X: the role of p12 and p8 encoded by the second and third open reading frames of the triple gene block. Mol. Plant-Microbe Interact. 14, 1158–1167.

Turner, D.R., Joyce, L.E., Butler, P.J., 1988. The Tobacco Mosaic Virus assembly origin RNA. Functional characteristics defined by directed mutagenesis. J. Mol. Biol. 203, 531–547.

Vaewhongs, A.A., Lommel, S.A., 1995. Virion formation is required for the long-distance movement of red clover necrotic mosaic virus in movement protein transgenic plants. Virology 212, 607–613.

Verchot-Lubicz, J., 2005. A new cell-to-cell transport model for Potexviruses. Mol. Plant-Microbe Interact. 18, 283–290.

Voinnet, O., 2005. Non-cell autonomous RNA silencing. FEBS Lett. 579, 5858–5871.

Voinnet, O., Vain, P., Angell, S., Baulcombe, D.C., 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell 95, 177–187.

Voinnet, O., Lederer, C., Baulcombe, D.C., 2000. A viral movement protein prevents spread of the gene silencing signal in *Nicotiana benthamiana*. Cell 103, 157–167.

Waigmann, E., Ueki, S., Trutnyeva, K., Citovsky, V., 2004. The ins and outs of nondestructive cell-to-cell and systemic movement of plant viruses. Crit. Rev. Plant Sci. 23, 195–250.

Wei, N., Hacker, D.L., Morris, T.J., 1992. Characterization of an internal element in turnip crinkle virus RNA involved in both coat protein binding and replication. Virology 190, 346–355.

White, K.A., Bancroft, J.B., Mackie, G.A., 1991. Defective RNAs of Clover Yellow Mosaic Virus encode nonstructural/coat protein fusion products. Virology 183, 479–486.

Xoconostle-Cázares, B., Xiang, Y., Ruiz-Medrano, R., Wang, H.-L., Monzer, J., Yoo, B.-C., McFarland, K.C., Franceschi, V.R., Lucas, W.J., 1999. Plant paralog to viral movement protein that potentiates transport of mRNA into the phloem. Science 283, 94–98.

Yang, Y., Ding, B., Baulcombe, D.C., Verchot, J., 2000. Cell-to-cell movement of the 25K protein of potato virus X is regulated by three other viral proteins. Mol. Plant-Microbe Interact. 13, 599–605.

Yeh, T.Y., Lin, B.Y., Chang, Y.C., Hsu, Y.-H., Lin, N.-S., 1999. A defective RNA associated with Bamboo Mosaic Virus and the possible common mechanisms for RNA recombination in potexviruses. Virus Genes 18, 121–128.

Yoo, B.C., Kragler, F., Varkonyi-Gasic, E., Haywood, V., Archer-Evans, S., Lee, Y.M., Lough, T.J., Lucas, W.J., 2004. A systemic small RNA signaling system in plants. Plant Cell 16, 1979–2000.

Zamore, P.D., Haley, B., 2005. Ribo-gnome: the big world of small RNAs. Science 309, 1519–1524.

Zamyatnin Jr., A.A., Solovyev, A.G., Sablina, A.A., Agranovsky, A.A., Katul, L., Vetten, H.J., Schiemann, J., Hinkkanen, A.E., Lehto, K., Morozov, S.Y., 2002. Dual-colour imaging of membrane protein targeting directed by poa semilatent virus movement protein TGBp3 in plant and mammalian cells. J. Gen. Virol. 83, 651–662.

Zamyatnin Jr., A.A., Solovyev, A.G., Savenkov, E.I., Germundsson, A., Sandgren, M., Valkonen, J.P., Morozov, S.Y., 2004. Transient coexpression 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.

Zhong, W., Dasgupta, R., Rueckert, R., 1992. Evidence that the packaging signal for nodaviral RNA2 is a bulged stem–loop. Proc. Natl. Acad. Sci. U.S.A. 89, 11146–11150.

Zhu, Y., Green, L., Woo, Y.M., Owens, R., Ding, B., 2001. Cellular basis of potato spindle tuber viroid systemic movement. Virology 279, 69–77.

Zhu, Y., Qi, Y., Xun, Y., Owens, R., Ding, B., 2002. Movement of potato spindle tuber viroid reveals regulatory points of phloem-mediated RNA traffic. Plant Physiol. 130, 138–146.