Journal of General Virology (2013), **94**, 677–681 DOI 10.1099/vir.0.048793-0

Short Communication

Systemic transport of *Alfalfa mosaic virus* can be mediated by the movement proteins of several viruses assigned to five genera of the 30K family

Thor V. M. Fajardo,[1] Ana Peiró,[2] Vicente Pallás[2] and Jesús Sánchez-Navarro[2]

Correspondence
Jesús Sánchez-Navarro
jesanche@ibmcp.upv.es

[1]Embrapa Uva e Vinho, Rua Livramento, 515. Bento Gonçalves-RS, CEP 95700-000, Brazil

[2]Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Avenida de los Naranjos s/n, 46022 Valencia, Spain

Received 15 October 2012
Accepted 5 November 2012

We previously showed that the movement protein (MP) gene of *Alfalfa mosaic virus* (AMV) is functionally exchangeable for the cell-to-cell transport of the corresponding genes of *Tobacco mosaic virus* (TMV), *Brome mosaic virus*, *Prunus necrotic ringspot virus*, *Cucumber mosaic virus* and *Cowpea mosaic virus*. We have analysed the capacity of the heterologous MPs to systemically transport the corresponding chimeric AMV genome. All MPs were competent in systemic transport but required the fusion at their C terminus of the coat protein-interacting C-terminal 44 aa (A44) of the AMV MP. Except for the TMV MP, the presence of the hybrid virus in upper leaves correlated with the capacity to move locally. These results suggest that all the MPs assigned to the 30K superfamily should be exchangeable not only for local virus movement but also for systemic transport when the A44 fragment is present.

To establish a systemic infection, plant viruses must invade the adjacent cells via the cell-wall connections known as plasmodesmata, the so-called cell-to-cell transport (Lucas, 2006; Fernandez-Calvino *et al.*, 2011), and reach distal parts of the plant through the vascular tissue, a process denominated systemic transport (Carrington *et al.*, 1996; Lazarowitz & Beachy, 1999; Waigmann *et al.*, 2004; Ueki & Citovsky, 2007; Pallás *et al.*, 2011). For this purpose, the viruses express one or a few movement proteins (MPs) to support virus transport. MPs can determine host specificity (Waigmann *et al.*, 2007) and in some instances can influence viral pathogenicity (Pallás & García, 2011). Viral MPs facilitate the cell-to-cell transport of the virus by different mechanisms, permitting the transport of ribonucleoprotein complexes between MP and viral RNA [e.g. *Tobacco mosaic virus* (TMV); Waigmann *et al.*, 2007], plus the coat protein (CP) [*Cucumber mosaic virus* (CMV) or *Alfalfa mosaic virus* (AMV)] or virions particles (Ritzenthaler & Hofmann, 2007). In spite of the clear differences observed among the three transport mechanisms, a large number of these MPs have been assigned to the 30K superfamily (Melcher, 2000).

Systemic transport implies the entry into and the exit from the vascular tissue, and consequently, the infection of different cell types associated with it (see Ueki & Citovsky, 2007 and Pallás *et al.*, 2011 for recent reviews). The capacity of plant viruses to reach vascular tissue requires not only the use of MPs, but also the involvement of other viral proteins that can be related to the suppression of plant defences (e.g. silencing suppressors), protein translation (e.g. VPg) (Rajamäki & Valkonen, 2002), viral RNA-dependent RNA replication (Traynor *et al.*, 1991) or the presence of the CP (Ueki & Citovsky, 2007; Bol, 2008). AMV is the type member of the genus *Alfamovirus* and virus particles are required for its systemic transport (Herranz *et al.*, 2012; Sánchez-Navarro & Bol, 2001; Tenllado & Bol, 2000). We previously reported that the MP of AMV is functionally exchangeable with different MPs assigned to the 30K superfamily, allowing the cell-to-cell transport of the corresponding chimera constructs (Sánchez-Navarro *et al.*, 2006, 2010; Sánchez-Navarro & Bol, 2001). Except for the TMV MP, the remaining heterologous MPs require fusion at their C termini with the C-terminal 44 amino acids of the AMV MP (A44), which is responsible for interacting with the cognate CP (Sánchez-Navarro *et al.*, 2006). The present work analyses the capacity of several MPs of the 30K superfamily to support the systemic transport of chimeric AMV RNA 3, including MPs representative of the different cell-to-cell transport mechanisms.

We quantified the cell-to-cell transport of the AMV RNA 3 chimera modified to express GFP and carrying the previously described heterologous MPs (Sánchez-Navarro *et al.*, 2006). An analysis of the replication rates in P12 protoplasts showed that the chimera constructs accumulated at comparable levels (Sánchez-Navarro *et al.*, 2006). First, T7 transcripts from the AMV RNA 3 chimera constructs carrying the GFP gene and the corresponding

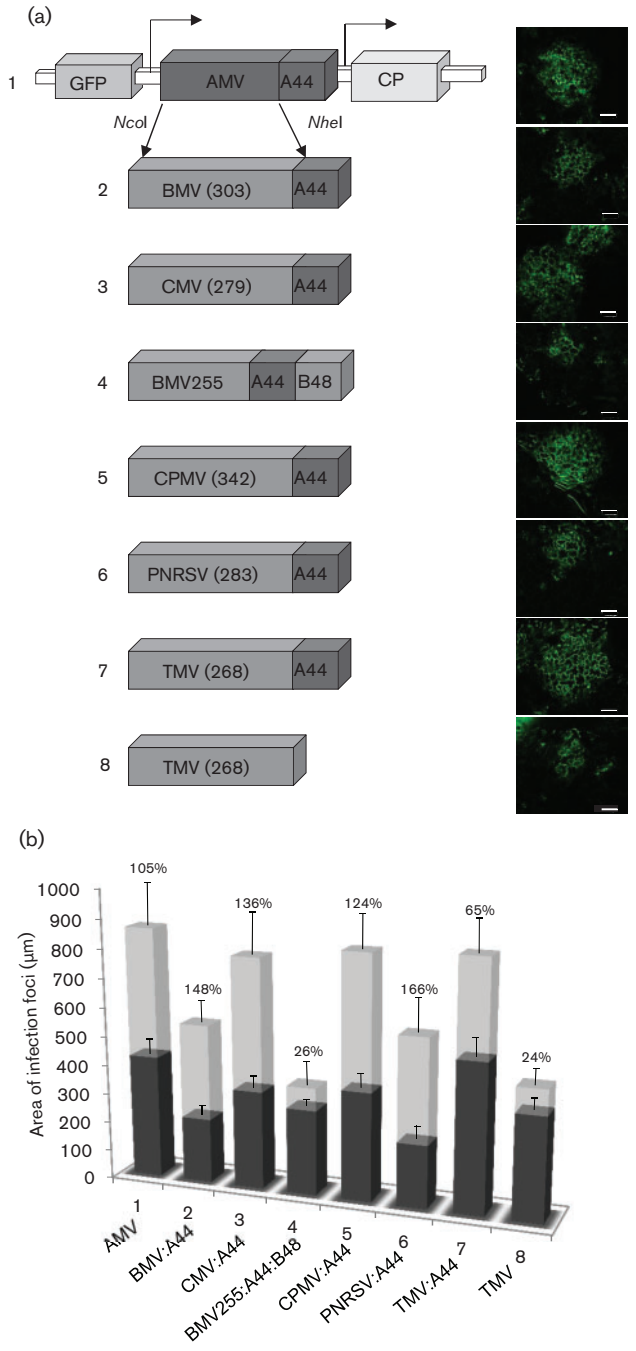

Fig. 1. Analysis of the cell-to-cell transport of the hybrid AMV RNA 3 in which its MP gene was exchanged with the corresponding genes of different viruses. (a) Schematic representation showing the GFP/AMV/CP and the AMV RNA 3 derivatives. The MPs analysed correspond to BMV (2, 4), CMV (3), CPMV (5), PNRSV (6) and TMV (7, 8). The C-terminal 44 and 48 aa fragments of the AMV and BMV MP are indicated as A44 and B48, respectively. Numbers in boxes represent the total amino acid residues of the corresponding MP. The *Nco*I and *Nhe*I restriction sites used to exchange the MP gene are indicated. Images on the right correspond to representative pictures of the size of infection foci observed on inoculated P12 leaves. Fluorescence was monitored with a confocal laser scanning microscope at 1 and 2 days p.i. Bars, 200 μm. (b) Graph showing the mean area of 50 independent infection foci resulting from inoculation with transcripts originating from the constructs shown in (a). Dark grey bars, 1 day p.i.; light grey bars, 2 days p.i. Error bars represent SD. Percentages show increases in the areas of infection from day 1 to day 2.

MP gene of *Prunus necrotic ringspot virus* (PNRSV : A44), CMV (CMV : A44), *Cowpea mosaic virus*, (CPMV : A44), *Brome mosaic virus* (BMV : A44), BMV with the A44 fused before its C-terminal 48 aa (BMV : A44 : B48) and TMV with or without the A44 fragment (TMV:A44 and TMV, respectively) were inoculated on transgenic tobacco plants constitutively expressing the AMV P1 and P2 protein (P12 plants; Taschner *et al.*, 1991). Fig. 1(b) shows the mean area of 50 infection foci at 1 and 2 days post-inoculation (p.i.). The 2 days p.i. results grouped constructs into three clusters, each with a different mean infection foci size: around 800 μm (AMV, CMV : A44, CPMV : A44 and TMV : A44), 600 μm (PNRSV : A44 and BMV : A44) and 400 μm (BMV : A44 : B48 and TMV). Interestingly, the absence of the A44 fragment (TMV construct) or its location inside the heterologous MP (BMV : A44 : B48) negatively affects the cell-to-cell transport (compare TMV : A44 versus TMV in Fig. 1b) with an area increment at 2 days p.i. considerably smaller than that observed for the rest of the constructs (24–26 % versus 65–166 %).

In the next step, we analysed the capacity of the heterologous MPs to support the systemic transport of AMV RNA 3. For this purpose, we modified a wild-type AMV RNA 3, since the RNA 3 derivatives carrying the GFP reporter gene do not move systemically in P12 tobacco plants (Sánchez-Navarro *et al.*, 2001). All the heterologous MPs were introduced into AMV RNA 3 (plasmid pAL3NcoP3 in Van der Vossen *et al.*, 1993) by exchanging the *Nco*I–*Pst*I fragment. RNA accumulation levels of the different AMV RNA 3 hybrids were first analysed in P12 protoplasts as described previously (Sánchez-Navarro *et al.*, 2010). Chimeric RNA 3 and 4 accumulated at comparable levels to AMV wild-type RNA 3 and 4 (lanes 2–6 versus lane 1 in Fig. 2b), except for the RNA 3 of the AMV constructs carrying the MP of TMV, either fused or not fused to the A44 fragment, which was significantly reduced (10 %, lanes 7 and 8 versus lane 1 in Fig. 2b). The accumulation of all the RNA 3 derivatives was then analysed in the inoculated and upper leaves of P12 plants by tissue printing of petioles, in which a positive hybridization signal, probably representing the capacity of the virus to infect the tissue adjacent to the phloem sieve elements, was always correlated with the presence of the virus in the corresponding leaf, as described previously (Más & Pallás, 1995; Sánchez-Navarro *et al.*, 2010). The tissue printing results (Fig. 2c) allow us to discern three different patterns according to the detection of a positive hybridization signal in: (i) all the inoculated and upper leaves (AMV, CMV : A44, CPMV : A44 and PNRSV : A44),

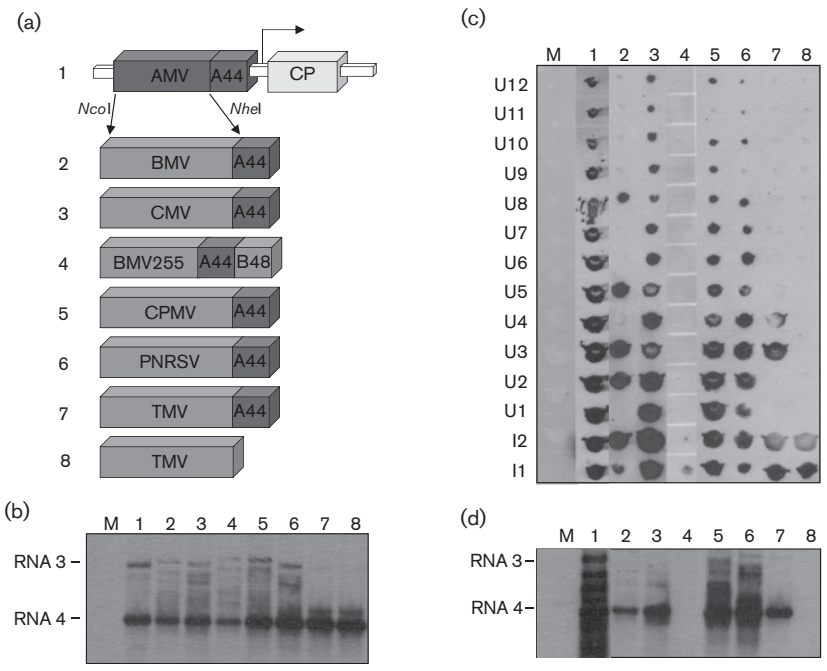

Fig. 2. Analysis of the replication and systemic transport of the AMV RNA 3 hybrids. (a) Schematic representation showing the AMV RNA 3 wild-type and its chimeras. The MP genes exchanged in the AMV RNA 3 are indicated in Fig. 1. (b) Northern blot analysis of the accumulation of the AMV RNA 3 and 4 chimeras in P12 protoplasts. (c) Tissue printing analysis of P12 plants inoculated with the AMV RNA 3 derivatives. Plants were analysed at 14 days p.i. by printing the transversal section of the corresponding petiole from inoculated and upper leaves (I and U, respectively). The position of each leaf is indicated by numbers, which correspond to the position of the leaves in the plant from the lower to the upper part. (d) Northern blot analysis of a mixture of total RNA extracted from the U2, U3 and U4 upper leaves. M, mock inoculated plant. Numbers at the top of each lane correspond to the constructs represented in (a). In all cases, the blots were hybridized with an AMV probe complementary to the 3′-UTR.

(ii) the inoculated leaves and some upper leaves (BMV : A44 and TMV : A44) and (iii) only the inoculated leaves (BMV : A44 : B48 and TMV). The accumulation of viral RNAs in the petioles of inoculated (not shown) or upper leaves showing positive hybridization signals by tissue printing was later confirmed by Northern blot analysis (Fig. 2d). The results shown in Fig. 2 revealed that all the analysed MPs carrying the A44 fragment fused at their C termini are able to support the systemic transport of the AMV RNA 3. Except for the TMV construct, all the AMV RNA 3 chimeras showing large infection foci on the inoculated leaves were able to infect all the upper leaves (CMV, CPMV and AMV). This result strongly suggests that an efficient cell-to-cell transport gives an advantage to

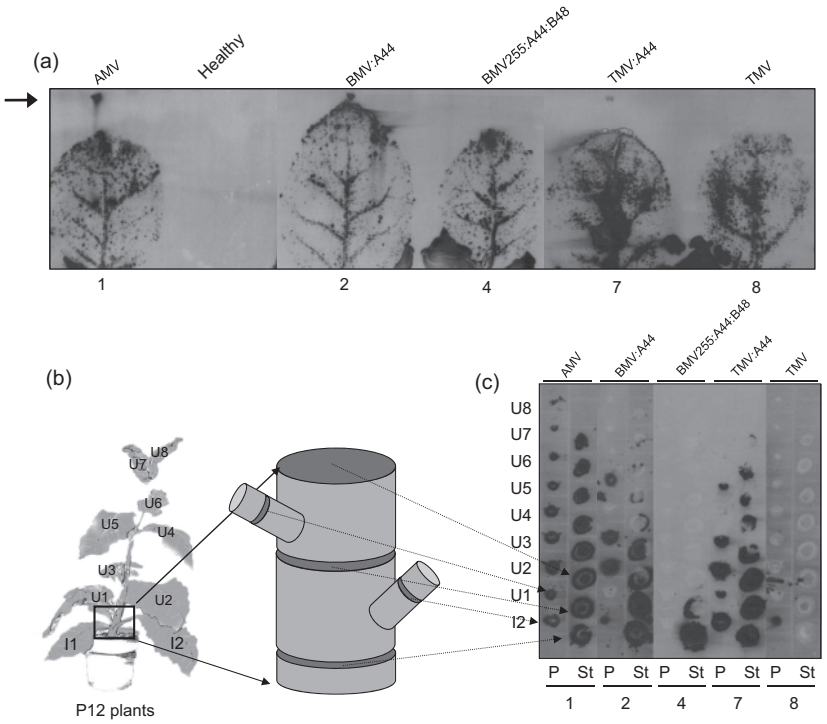

Fig. 3. Tissue printing analysis of AMV RNA 3 derivatives affected in systemic transport. P12 plants were inoculated with transcripts of AMV RNA 3 wild-type (1) or hybrids carrying the MP gene of BMV (2 and 4) and TMV (7 and 8) represented in Fig. 2(a). (a) Tissue printing analysis of the inoculated leaves of P12 plants at 7 days p.i. The arrow indicates the printing of the transverse section of the corresponding petiole. (b) Schematic representation of the localization of all the analysed leaves and the distribution of the transversal sections of petioles and stems. (c) Tissue printing analysis of the P12 plants at 14 days p.i. by printing transversal sections of all petioles and the stem around them. The hybridization was performed as described for Fig. 2. I, inoculated leaf; P, petiole; St, stem; U, upper leaf.

the pathogen, which could help avoid the plant defence mechanisms (e.g. silencing, pathogenesis-related proteins, hypersensitive response, etc.). Indeed, in some well-characterized plant–virus interactions, the capacity to reach the upper tissue has been associated with a successful blockage of the RNA silencing-mediated plant defence barriers (Cao *et al.*, 2010; Hamilton *et al.*, 2002; Schwach *et al.*, 2005; Wintermantel *et al.*, 1997; Yelina *et al.*, 2002). However, it was not possible to apply this idea to the TMV : A44 construct, since the infection foci observed on the inoculated leaves were similar to those observed for other AMV chimeras that were able to infect all the upper leaves. This result clearly reveals that despite the TMV MP being very efficient at supporting the cell-to-cell transport of the AMV RNA 3 chimera, it is very inefficient at invading vascular tissue. The observation that the TMV construct is also competent in cell-to-cell transport indicates that the MP transports viral RNA without any interaction with the AMV CP. In this scenario, it is tempting to speculate that the TMV : A44 MP mainly transports non-encapsidated viral RNA, which allows a very efficient, local transport. However, it presents an inefficient systemic movement because the presence of AMV virus particles is critical for reaching the distal parts of the plant. The group of AMV constructs showing medium infection foci on inoculated leaves (600 μm; BMV : A44 and PNRSV : A44) rendered two different systemic infection patterns, which were differentiated in terms of their capacity to reach all the upper leaves (PNRSV : A44; Fig. 2c, lane 6) or only part of them (BMV : A44; Fig. 2c, lane 2). This result clearly indicates that AMV chimeras with reduced cell-to-cell transport are still able to infect all the upper leaves. The differences observed between both constructs can be attributed to the greater compatibility between the PNRSV and AMV viruses (Aparicio *et al.*, 2003; Codoñer *et al.*, 2005; Sánchez-Navarro & Pallás, 1997; Sánchez-Navarro *et al.*, 1997). To further characterize the AMV constructs that are affected in systemic transport, we decided to perform a more precise tissue printing analysis by checking not only the petiole, but also the inoculated leaf and the stem just above and below the corresponding petiole (Fig. 3). First we observed that at 7 days p.i. all constructs analysed rendered a comparable hybridization signal in the inoculated leaf (Fig. 3a); meanwhile, no signal at all was observed in the transverse section of the corresponding petiole for the constructs that do not move systemically (Fig. 3a, panels 4 and 8). At 14 days p.i. we observed positive hybridization signals in all the stem sections for the AMV wild-type, covering the full ring and indicating the presence of viral RNA in all phloem tissue. However, the constructs that moved only to some of the upper leaves (BMV : A44 and TMV : A44) rendered a strong stem hybridization signal close to the inoculated leaves that decreased in the upper part of the plant, where the hybridization signal was observed in only part of the cross-section (Fig. 3c, stem lanes 2 and 7). This result indicates that both constructs are able to reach the vascular tissue less efficiently than the AMV wild-type. A less efficient transport through the vascular system would not allow the virus to reach some of the upper leaves (e.g. U1 and U4 for petiole lane 2, and U4 for petiole lane 7), which have already undergone the sink–source transition, as has been described in other virus–host interactions (Cheng *et al.*, 2000; Más & Pallás, 1996). For the constructs that do not move systemically, we observed two different patterns on the stem sections: (i) the BMV255 : A44 : B48 chimera shows a clear hybridization signal only in the stem sections around the inoculated leaf (Fig. 3c, lane 4), and (ii) the TMV construct shows no hybridization signal at all in the stem (Fig. 3c, lane 8). Regarding the hybridization signal observed with the BMV255 : A44 : B48 construct on the border of the stem section, we can conclude that this construct is competent enough to reach vascular tissue, but it is quite likely that a delay in reaching it does not permit the establishment of a systemic infection. For TMV, we observed the opposite situation, in which the lack of the A44 fragment compromises the accession of the virus to the phloem. In line with this, we have recently reported that virus particles and the A44 fragment are essential for the systemic transport of an AMV chimera carrying the MP of *Cauliflower mosaic virus* (Sánchez-Navarro *et al.*, 2010).

In summary, we have shown that the MPs analysed in the present work are competent enough to systemically transport the AMV chimera constructs to the distal parts of the plant when the last 44 aa of the AMV MP were fused at their C termini. These results allow us to suggest the idea that all the MPs of the 30K family are functionally exchangeable for both the local and systemic transport of AMV, irrespective of the virus, the model used for the local transport (e.g. MP of TMV or CPMV) or the pathway used to reach the plasmodesmata (e.g. MP of TMV or *Grapevine fanleaf virus*; Sánchez-Navarro *et al.*, 2010). In addition, this work also shows that an inefficient cell-to-cell transport compromises systemic invasion, permitting the postulation of the idea that a minimal cell-to-cell speed is required to reach the upper part of the plant as formerly reported for other viruses (Deom *et al.*, 1994).

Acknowledgements

We thank L. Corachán for her excellent technical assistance. This work was supported by the Spanish granting agency DGICYT via grant BIO2011-25018 and by the Generalitat Valenciana via grant PROMETEO 2011-003.

References

Aparicio, F., Vilar, M., Perez-Payá, E. & Pallás, V. (2003). The coat protein of prunus necrotic ringspot virus specifically binds to and regulates the conformation of its genomic RNA. *Virology* **313**, 213–223.

Bol, J. F. (2008). Role of capsid proteins. *Methods Mol Biol* **451**, 21–31.

Cao, M., Ye, X., Willie, K., Lin, J., Zhang, X., Redinbaugh, M. G., Simon, A. E., Morris, T. J. & Qu, F. (2010). The capsid protein of *Turnip crinkle*

virus overcomes two separate defense barriers to facilitate systemic movement of the virus in *Arabidopsis*. *J Virol* **84**, 7793–7802.

Carrington, J. C., Kasschau, K. D., Mahajan, S. K. & Schaad, M. C. (1996). Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* **8**, 1669–1681.

Cheng, N. H., Su, C. L., Carter, S. A. & Nelson, R. S. (2000). Vascular invasion routes and systemic accumulation patterns of tobacco mosaic virus in *Nicotiana benthamiana*. *Plant J* **23**, 349–362.

Codoñer, F. M., Cuevas, J. M., Sánchez-Navarro, J. A., Pallás, V. & Elena, S. F. (2005). Molecular evolution of the plant virus family Bromoviridae based on RNA3-encoded proteins. *J Mol Evol* **61**, 697–705.

Deom, C. M., He, X. Z., Beachy, R. N. & Weissinger, A. K. (1994). Influence of heterologous tobamovirus movement protein and chimeric-movement protein genes on cell-to-cell and long-distance movement. *Virology* **205**, 198–209.

Fernandez-Calvino, L., Faulkner, C., Walshaw, J., Saalbach, G., Bayer, E., Benitez-Alfonso, Y. & Maule, A. (2011). *Arabidopsis* plasmodesmal proteome. *PLoS ONE* **6**, e18880.

Hamilton, A., Voinnet, O., Chappell, L. & Baulcombe, D. (2002). Two classes of short interfering RNA in RNA silencing. *EMBO J* **21**, 4671–4679.

Herranz, M. C., Pallás, V. & Aparicio, F. (2012). Multifunctional roles for the N-terminal basic motif of *Alfalfa mosaic virus* coat protein: nucleolar/cytoplasmic shuttling, modulation of RNA-binding activity, and virion formation. *Mol Plant Microbe Interact* **25**, 1093–1103.

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

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

Más, P. & Pallás, V. (1995). Non-isotopic tissue-printing hybridization: a new technique to study long-distance plant virus movement. *J Virol Methods* **52**, 317–326.

Más, P. & Pallás, V. (1996). Long-distance movement of cherry leaf roll virus in infected tobacco plants. *J Gen Virol* **77**, 531–540.

Melcher, U. (2000). The '30K' superfamily of viral movement proteins. *J Gen Virol* **81**, 257–266.

Pallás, V. & García, J. A. (2011). How do plant viruses induce disease? Interactions and interference with host components. *J Gen Virol* **92**, 2691–2705.

Pallás, V., Genovés, A., Sánchez-Pina, M. A. & Navarro, J. A. (2011). Sytemic movement of viruses via the plant phloem. In *Recent Advances in Plant Virology*, pp. 75–101. Edited by C. Caranta, M. G. Aranda, M. Tepfer & J. J. López-Moya. Norfolk, UK: Caister Academic Press.

Rajamäki, M. L. & Valkonen, J. P. (2002). Viral genome-linked protein (VPg) controls accumulation and phloem-loading of a potyvirus in inoculated potato leaves. *Mol Plant Microbe Interact* **15**, 138–149.

Ritzenthaler, C. & Hofmann, C. (2007). Tubule-guided movement of plant viruses. In *Viral Transport in Plants*, pp. 63–83. Edited by E. Waigmann & M. Heinlein. Berlin: Springer.

Sánchez-Navarro, J. A. & Bol, J. F. (2001). Role of the *Alfalfa mosaic virus* movement protein and coat protein in virus transport. *Mol Plant Microbe Interact* **14**, 1051–1062.

Sánchez-Navarro, J. A. & Pallás, V. (1997). Evolutionary relationships in the ilarviruses: nucleotide sequence of prunus necrotic ringspot virus RNA 3. *Arch Virol* **142**, 749–763.

Sánchez-Navarro, J. A., Reusken, C. B. E. M., Bol, J. F. & Pallás, V. (1997). Replication of alfalfa mosaic virus RNA 3 with movement and coat protein genes replaced by corresponding genes of Prunus necrotic ringspot ilarvirus. *J Gen Virol* **78**, 3171–3176.

Sánchez-Navarro, J., Miglino, R., Ragozzino, A. & Bol, J. F. (2001). Engineering of *Alfalfa mosaic virus* RNA 3 into an expression vector. *Arch Virol* **146**, 923–939.

Sánchez-Navarro, J. A., Carmen Herranz, M. & Pallás, V. (2006). Cell-to-cell movement of *Alfalfa mosaic virus* can be mediated by the movement proteins of Ilar-, bromo-, cucumo-, tobamo- and comoviruses and does not require virion formation. *Virology* **346**, 66–73.

Sánchez-Navarro, J., Fajardo, T., Zicca, S., Pallás, V. & Stavolone, L. (2010). *Caulimoviridae* tubule-guided transport is dictated by movement protein properties. *J Virol* **84**, 4109–4112.

Schwach, F., Vaistij, F. E., Jones, L. & Baulcombe, D. C. (2005). An RNA-dependent RNA polymerase prevents meristem invasion by potato virus X and is required for the activity but not the production of a systemic silencing signal. *Plant Physiol* **138**, 1842–1852.

Taschner, P. E., Van der Kuyl, A. C., Neeleman, L. & Bol, J. F. (1991). Replication of an incomplete alfalfa mosaic virus genome in plants transformed with viral replicase genes. *Virology* **181**, 445–450.

Tenllado, F. & Bol, J. F. (2000). Genetic dissection of the multiple functions of alfalfa mosaic virus coat protein in viral RNA replication, encapsidation, and movement. *Virology* **268**, 29–40.

Traynor, P., Young, B. M. & Ahlquist, P. (1991). Deletion analysis of brome mosaic virus 2a protein: effects on RNA replication and systemic spread. *J Virol* **65**, 2807–2815.

Ueki, S. & Citovsky, V. (2007). Spread throughout the plant: systemic transport of viruses. In *Viral Transport in Plants*, pp. 85–118. Edited by E. Waigmann & M. Heinlein. Berlin: Springer.

Van der Vossen, E. A., Neeleman, L. & Bol, J. F. (1993). Role of the 5′ leader sequence of alfalfa mosaic virus RNA 3 in replication and translation of the viral RNA. *Nucleic Acids Res* **21**, 1361–1367.

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.

Waigmann, E., Curin, M. & Heinlein, M. (2007). Tobacco mosaic virus – a model for macromolecular cell-to-cell spread. In *Viral Transport in Plants*, pp. 29–62. Edited by E. Waigmann & M. Heinlein. Berlin: Springer.

Wintermantel, W. M., Banerjee, N., Oliver, J. C., Paolillo, D. J. & Zaitlin, M. (1997). Cucumber mosaic virus is restricted from entering minor veins in transgenic tobacco exhibiting replicase-mediated resistance. *Virology* **231**, 248–257.

Yelina, N. E., Savenkov, E. I., Solovyev, A. G., Morozov, S. Y. & Valkonen, J. P. (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.