

## *Caulimoviridae* Tubule-Guided Transport Is Dictated by Movement Protein Properties<sup>∇</sup>

Jesús Sánchez-Navarro,<sup>1</sup>§ Thor Fajardo,<sup>2</sup>§ Stefania Zicca,<sup>3,4</sup> Vicente Pallás,<sup>1</sup> and Livia Stavolone<sup>4\*</sup>

*Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Valencia, Spain<sup>1</sup>; Embrapa Uva e Vinho, Rua Livramento, Bento Gonçalves, Rio Grande do Sul, Brazil<sup>2</sup>; DPPMA, Università di Bari, Bari, Italy<sup>3</sup>; and Istituto di Virologia Vegetale, CNR, Bari, Italy<sup>4</sup>*

Received 4 December 2009/Accepted 27 January 2010

**Plant viruses move through plasmodesmata (PD) either as nucleoprotein complexes (NPCs) or as tubule-guided encapsidated particles with the help of movement proteins (MPs). To explore how and why MPs specialize in one mechanism or the other, we tested the exchangeability of MPs encoded by DNA and RNA virus genomes by means of an engineered alfalfa mosaic virus (AMV) system. We show that *Caulimoviridae* (DNA genome virus) MPs are competent for RNA virus particle transport but are unable to mediate NPC movement, and we discuss this restriction in terms of the evolution of DNA virus MPs as a means of mediating DNA viral genome entry into the RNA-trafficking PD pathway.**

Following virus entry and replication, successful infection of a host requires viral spread to distal parts of the organism through the vascular tissue. In plants, virus movement involves mostly symplastic trafficking of different viral components through the connections of plasmodesmata (PD) (13). With this aim, plant viruses encode one or more movement proteins (MPs), which allow viral genomes to cross the host cell wall by altering the size exclusion limit (SEL) or the structure of PD (6, 11). Plant viruses have evolved distinct mechanisms to move their genomes within the host. These mechanisms can be grouped into two general strategies: one in which the genome is transported in the form of a nucleoprotein complex (NPC) and another in which nucleic acids are encapsidated and move as virus particles. In both cases, besides altering PD SEL, MPs are involved either in NPC assembly or in forming tubules traversing modified PD and helping transport of either NPC or virions to the neighboring cell. Within these two major strategies, there exists a wide range of variability in terms of the number and type of viral and host proteins helping MPs to mediate virus spread within the host (11).

In spite of such variability, several different MPs have been classified into a 30K superfamily; these MPs, from 20 genera including both RNA and DNA genome viruses, are structurally related to the 30-kDa MP of *Tobacco mosaic virus* (TMV), independent of the movement strategy followed (14). Members of this family have a common core of predicted secondary structure elements ( $\alpha$ -helices and  $\beta$ -elements) containing a nucleic acid binding domain. Distinct MPs belong to this family, including several tubule-forming MPs, although these are phylogenetically separated from the other members (14). Thus, 30K superfamily MPs are closely related, and some of them are functionally interchangeable in the viral context (2, 20). In

particular, MPs from five distinct genera with an RNA genome can successfully replace the corresponding gene of *Alfalfa mosaic virus* (AMV) (19), indicating that one or more basic and fundamental movement properties might be associated with the common 30K structural core.

Among all known plant viruses, only three viral families have evolved a DNA genome: *Geminiviridae*, *Caulimoviridae*, and *Nanoviridae* (6). One possible explanation for this restriction is that endogenous cell-to-cell transport via PD is specialized to use RNA as the communication and signaling molecule (12). To circumvent this restriction, and to allow the efficient exploitation of endogenous transport machineries, DNA genome viruses have evolved appropriate mechanisms involving their MPs. Interestingly, *Begomovirus* and *Caulimovirus* MPs also belong to the 30K superfamily discussed above (14). The MP encoded by *Cauliflower mosaic virus* (CaMV), the type member of *Caulimoviridae*, forms tubules that guide the movement of encapsidated virus via an indirect MP-virion interaction (16, 21), whereas geminivirus MPs selectively bind their genomes and transport them as NPCs (6, 9, 17). In this study, we investigated the evolutionary convergence of MPs encoded by DNA and RNA viruses by testing their exchangeability in the viral context.

**CaMV MP mediates cell-to-cell movement of AMV.** A system based on modified AMV (19, 24) was used to test whether CaMV MP could mediate RNA virus transport. This system allows monitoring of cell-to-cell and long-distance transport of an engineered AMV RNA 3 transcript encoding, in addition to viral MP and coat protein (CP), green fluorescent protein (GFP). This hybrid transcript leads to virus infection when inoculated onto transgenic tobacco plants expressing AMV P1 and P2 polymerases (P12 plants).

As previously performed with other 30K superfamily members (19), the RNA-transcribing pGFP/MP/CP clone was modified to replace the AMV MP gene with the CaMV MP gene obtained from the pCa37 clone (10), either with or without the region corresponding to the C-terminal 44 residues of AMV MP (A44) (Fig. 1A). The A44 domain (amino acids 256 to 300) is dispensable for tubule formation, cell-to-cell movement, and systemic transport of AMV (18) but interacts specifically with

\* Corresponding author. Mailing address: Istituto di Virologia Vegetale, Consiglio Nazionale delle Ricerche, Via Amendola 165/A, 70126 Bari, Italy. Phone: 390805442545. Fax: 390805442911. E-mail: l.stavolone@ba.ivv.cnr.it.

§ These authors contributed equally to this work.

<sup>∇</sup> Published ahead of print on 3 February 2010.

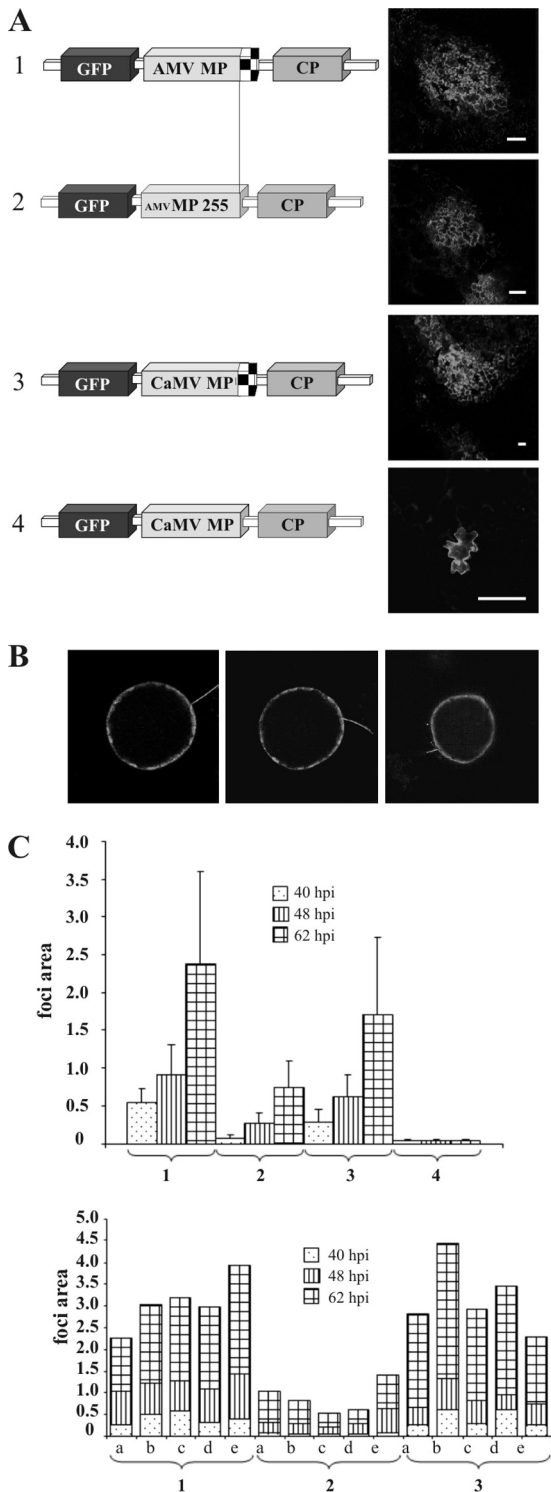


FIG. 1. Detection of infection foci in P12 plants two days after inoculation with a hybrid AMV RNA 3 in which the AMV movement protein (MP) gene was exchanged for the MP gene of CaMV. (A) Schematic representation of chimeric AMV RNA 3 (construct 1) and mutants (constructs 2 to 4). GFP, CP, and MP coding regions are depicted by boxes; the brick pattern represents the AMV MP C-terminal 44 amino acids. Fluorescence images illustrate chimeric RNA 3 distributions in P12 plants. Bar = 100  $\mu$ m. (B) Fluorescence images of P12 plant protoplasts infected with hybrid AMV RNA 3 expressing GFP-CaMV MP/CP (left panel), GFP-CaMV MP:A44/CP (middle panel), or GFP-CaMV

CP (19). Transcripts were inoculated onto P12 plants and protoplasts, and fluorescent signals were monitored by confocal laser scanning microscopy at 2 days postinoculation. Both GFP-CaMV MP/CP and GFP-CaMV MP:A44/CP formed tubules protruding from the protoplast surface (Fig. 1B, left and middle panels, respectively). As observed for all previously tested 30K superfamily members, only CaMV MP:A44 formed foci (Fig. 1A and C) that expanded with time (Fig. 1C, lower graph). No such foci formed with GFP-CaMV MP/CP. The requirement for A44 confirmed that, except for TMV (19) and AMV (Fig. 1A) (19) MPs, the MP-CP interaction is indispensable for exogenous MPs to mediate AMV transport. Removal of A44 also reduces movement efficiency of AMV MP (Fig. 1C, histograms 1 and 2 in both graphs); in fact, the rate of size increase of CaMV MP:A44 foci was 2- to 3-fold higher than that of AMV MP255 foci (Fig. 1C, histograms 3 and 2, respectively, in both graphs).

**Virus encapsidation is essential for CaMV MP-mediated movement.** The finding that MP-CP interaction is indispensable for AMV transport did not rule out the possibility that CaMV MP:A44 also mediates NPC movement. To examine the strategy used by CaMV MP to mediate AMV movement, pGFP/CaMVMP:A44/CP was further modified to express a shorter AMV CP (CP 206) lacking the C-terminal 14 amino acids. CP 206 is competent for cell-to-cell movement and RNA accumulation but not for virion encapsidation (19, 22). In contrast with all other previously tested MPs (19), CaMV MP was unable to transport nonencapsidated viral nucleic acids, which remained confined to single cells in the presence of CP 206 (Fig. 2A, image 2), although comparable levels of MP subgenomic RNA accumulated (Fig. 2B, lanes 1 and 2). This might indicate a distinct evolution for MPs involved in tubule-mediated virus transport. To further investigate this hypothesis, we replaced the wild-type (WT) AMV MP with the MP of *Grapevine fanleaf virus* (GFLV) (8), an RNA virus that moves from cell to cell in a tubule-guided fashion. We found that GFLV MP facilitated the formation of infection foci in conjunction with either WT AMV CP or CP 206 (Fig. 2A, images 3 and 4). This result is in agreement with previous studies with *Cowpea mosaic virus* (CPMV) MP (7, 19), which also directs the formation of tubules, and confirmed that tubule-forming MPs from RNA viruses mediate both NPC and virion transport of AMV.

These results provide biological support for the 30K superfamily classification by showing that its members are exchangeable beyond the movement strategy employed and indicate that tubule-guided transport is an additional, rather than alternative, feature developed by a group of these viruses, with the exception of CaMV, which apparently lacks, or has lost, the ability to move NPCs. We reasoned that limitation of CaMV

MP285:A44/CP (right panel). (C) Size expansion of infection foci formed upon inoculation in P12 plants of the four RNA 3 hybrids illustrated in panel A. The upper graph illustrates the average sizes of 20 different infection foci; the lower graph shows size evolution of 5 (a through e) randomly selected single foci. Focus area is measured in square millimeters. Bars represent standard deviations. hpi, hours postinoculation.

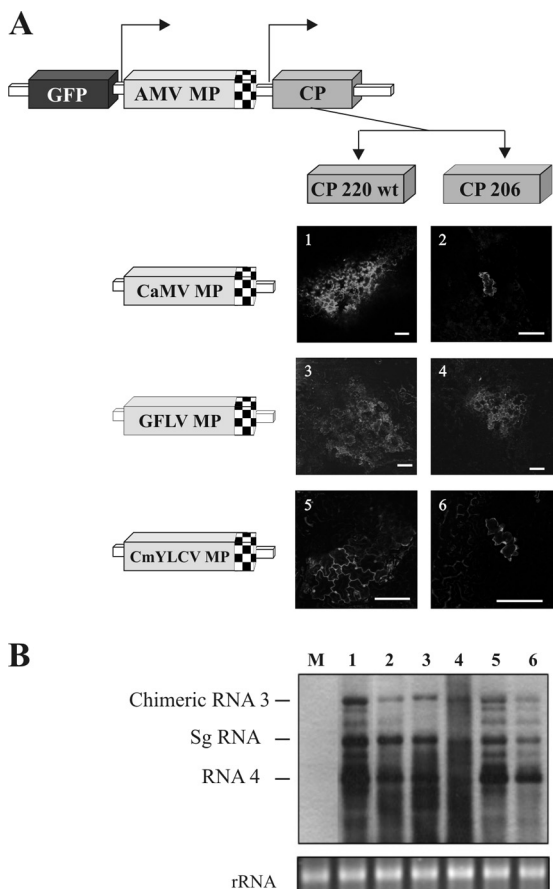


FIG. 2. Chimeric AMV RNA 3 accumulation and cell-to-cell transport in P12 plants. (A) Detection of fluorescent infection foci two days after inoculation with AMV RNA 3 derivatives obtained by exchange of AMV MP with that of CaMV (images 1 and 2), GFLV (images 3 and 4), or CmYLCV (images 5 and 6) MPs. A schematic representation shows the GFP/MP:A44/CP AMV RNA 3 derivatives as depicted in Fig. 1. Arrows above the boxes indicate the start of subgenomic RNAs. CP 220, AMV WT coat protein; CP 206, AMV deletion mutant coat protein. Bar = 100  $\mu$ m. (B) Northern blot analysis of chimeric RNA 3 derivative accumulation in P12 protoplasts. Total RNA extracted from protoplasts at 16 h postinoculation were glyoxylated, loaded onto a 1.5% agarose gel, and hybridized with a digoxigenin-labeled riboprobe complementary to the 3' UTR of RNA 3. Lane numbers correspond to the analyzed constructs shown in panel A. M, mock inoculation. The positions of the chimeric RNA 3, the first subgenomic RNA (Sg RNA), and RNA 4 are indicated.

MP to the tubule-guided movement strategy could ensure safe transport of the viral DNA genome through the non-cell-autonomous RNA-specific pathway. To explore this hypothesis, we extended our analysis to *Cestrum yellow leaf curling virus* (CmYLCV) and *Mungbean yellow mosaic virus* (MYMV) MPs. CmYLCV belongs to a distinct genus of *Caulimoviridae*, whereas MYMV is a member of the family *Geminiviridae*. Upon inoculation of GFP-CmYLCV A44/CP 206 transcript into P12 plants, fluorescent signal appeared but remained confined to single cells, unlike that produced with WT CP (Fig. 2A, images 5 and 6), indicating that CmYLCV MP is subject to the same restrictions as CaMV MP. MYMV MP was not functional in this system either with or without A44 addition (data not shown).

**Systemic transport of chimeric AMV RNA 3.** Accumulation of RNA 3 derivatives expressing CaMV, GFLV, and CmYLCV MPs (Fig. 3A) was analyzed in inoculated and systemically infected P12 leaves by tissue printing of petioles. All RNA 3 constructs tested were detected in inoculated leaves, with the intensity of the signal decreasing from inoculated leaves toward distal leaves (Fig. 3B). Derivative RNA 3 containing GFLV MP (Fig. 3B, lane 4) or CPMV MP (T. Fajardo et al., unpublished data) moved systemically at a speed similar to that of WT MP and accumulated to levels even higher than those of the WT. This indicates that RNA-encoded tubule-forming MPs are highly competent to mediate AMV movement in our system.

On the contrary, a lower efficiency of CaMV MP:A44 was indicated by the decreased intensity of the signal starting from the third systemically infected leaf (Fig. 3B, lane 2). The CmYLCV A44 derivative RNA 3 did not move out of inoculated leaves, suggesting even stronger restriction for CmYLCV MP (Fig. 3B, lane 3). Tissue printing results were confirmed by Northern blot analysis using the same riboprobe specific for the 3' untranslated region (UTR) of AMV RNA 3 (Fig. 3C).

**Implications for DNA virus transport.** The 30K superfamily phylogenetic tree places all tubule-forming MPs in a separate cluster (15), suggesting an important relationship between MP secondary structure and movement strategy. Our results and previous results (19) support this prediction, showing that all tested 30K family members encoded by an RNA virus mediate virus NPC movement, whereas only a subset have developed the additional feature of controlling tubule-guided transport. CaMV MP joined the tubule cluster but, despite the presence of an RNA binding domain (RBD) (1, 23), did not mediate viral NPC transport. Globplot software (version 2.3; EMBL [http://globplot.embl.de]) predicts a globular tertiary structure for MPs. We reasoned that the CaMV MP RBD could be buried in such a structure and thus be unavailable for AMV NPC formation. To explore this possibility, we modified AMV RNA 3 to express a GFP:CaMV MP:RBD:A44/CP transcript containing an additional RBD from AMV, CaMV, and *Prunus necrotic ringspot virus* (PNRSV) (5) MPs. None of these constructs was able to mediate NPC transport or, surprisingly, encapsidated virus movement. As the larger chimeric MP-RBD constructs could interfere with virion movement through tubules, we expressed a shorter tubule-competent construct, CaMV MP285-RBD (Fig. 1B, right panel) (21), similar in size to WT CaMV MP; however, this construct did not restore virion transport (data not shown). This indicates that movement inhibition cannot be a consequence of steric hindrance of the chimeric MP but is most probably a structural modification resulting from the RBD addition. While a number of reasons could explain why the addition of RBDs does not help NPC movement, inactivation of virion transport under such experimental conditions underscores the pivotal role played by the three-dimensional structure of CaMV MP. It is tempting to speculate that, in CaMV, this structure could have evolved to prevent NPC formation and thus mediate only encapsidated virus transport, thus avoiding direct access of the CaMV DNA genome to the plant cell-to-cell trafficking pathway specialized for RNA molecule traffic. With the same aim, geminivirus, moving as an NPC, evolved to have DNA genome molecules small enough to be restrictively selected by MP and elude detection by the RNA recognition system of the PD pathway



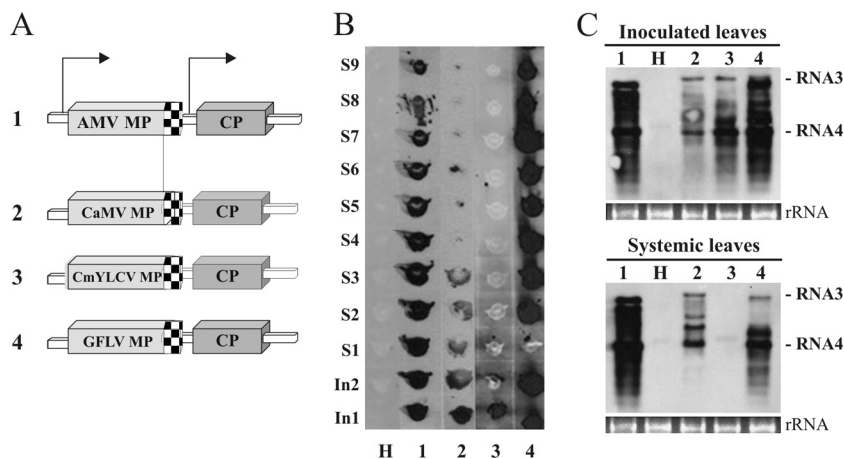


FIG. 3. Systemic movement of AMV RNA 3 derivatives in P12 plants. (A) Schematic representation of WT AMV RNA 3 (construct 1) and derivatives expressing the MPs of CaMV (construct 2), CmYLCV (construct 3), and GFLV (construct 4). CPs and MPs are depicted by boxes. The brick pattern represents the AMV MP C-terminal 44 amino acids. Arrows indicate the start of RNAs 3 and 4. (B) Tissue printing of petiole transversal sections of the two inoculated leaves (In1 and In2) and all available systemic leaves (S1 through S9) at 7 (inoculated leaves) or 14 (systemic leaves) days postinoculation with the RNA constructs represented in panel A. H, healthy plant. (C) Northern blot analysis of accumulation of WT AMV RNA 3 (lane 1) and derivatives (lanes 2, 3, and 4) in inoculated and systemic leaves. Samples consist of a mix of leaf tissue from the two inoculated leaves or from the S1, S2, and S3 systemic leaves. H, healthy plant. Positions of RNA 3 and RNA 4 are indicated on the right.

(3, 4, 9). A very restrictive evolution of geminivirus MP could explain the inability of MYMV MP to mediate AMV movement with and without the addition of RBDs (data not shown). We believe that MP structure has played an important role in the evolution of DNA virus-encoded MP specialization to mediate virus transport through the non-DNA-permissive plasmodesmata pathway. To provide further evidence of this, efforts to determine the structure of CaMV MP are under way.

This work was partially supported by grant BIO2008-03528 from the Spanish Ministry of Science and Innovation. The laboratories of V.P. and L.S. were jointly supported by Consiglio Nazionale delle Ricerche-Consejo Superior de Investigaciones Cientificas project 2004IT0028. T.F. was the recipient of a postdoctoral fellowship from CNPq (Brazil).

We thank Helen Rohnie and Thomas Hohn for critical reading of the manuscript.

#### REFERENCES

- Citovsky, V., D. Knorr, and P. Zambryski. 1991. Gene-I, a potential cell-to-cell movement locus of Cauliflower mosaic virus, encodes an RNA-binding protein. *Proc. Natl. Acad. Sci. U. S. A.* **88**:2476–2480.
- De Jong, W., and P. Ahlquist. 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.
- Gilbertson, R. L., M. Sudarshana, H. Jiang, M. R. Rojas, and W. J. Lucas. 2003. Limitations on geminivirus genome size imposed by plasmodesmata and virus-encoded movement protein: insights into DNA trafficking. *Plant Cell* **15**:2578–2591.
- Hehnle, S., C. Wege, and H. Jeske. 2004. Interaction of DNA with the movement proteins of geminiviruses revisited. *J. Virol.* **78**:7698–7706.
- Herranz, M. C., J. A. Sanchez-Navarro, A. Sauri, I. Mingarro, and V. Pallas. 2005. Mutational analysis of the RNA-binding domain of the Prunus necrotic ringspot virus (PNRSV) movement protein reveals its requirement for cell-to-cell movement. *Virology* **339**:31–41.
- Hull, R. 2002. *Matthews' plant virology*, 4th ed. Academic Press, San Diego, CA.
- Kasteel, D. T. J., M. C. Perbal, J. C. Boyer, J. Wellink, R. W. Goldbach, A. J. Maule, and J. W. M. vanLent. 1996. The movement proteins of cowpea mosaic virus and cauliflower mosaic virus induce tubular structures in plant and insect cells. *J. Gen. Virol.* **77**:2857–2864.
- Laporte, C., G. Vetter, A. M. Loudes, D. G. Robinson, S. Hillmer, C. Stussi-Garaud, and C. Ritzenthaler. 2003. Involvement of the secretory pathway and the cytoskeleton in intracellular targeting and tubule assembly of Grapevine fanleaf virus movement protein in tobacco BY-2 cells. *Plant Cell* **15**:2058–2075.
- Lazarowitz, S. G., and R. N. Beachy. 1999. Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *Plant Cell* **11**:535–548.
- Lebeurier, G., L. Hirth, T. Hohn, and B. Hohn. 1980. Infectivities of native and cloned DNA of cauliflower mosaic virus. *Gene* **12**:139–146.
- Lucas, W. J. 2006. Plant viral movement proteins: agents for cell-to-cell trafficking of viral genomes. *Virology* **344**:169–184.
- Lucas, W. J., B. C. Yoo, and F. Kragler. 2001. RNA as a long-distance information macromolecule in plants. *Nat. Rev. Mol. Cell Biol.* **2**:849–857.
- Maule, A. J. 2008. Plasmodesmata: structure, function and biogenesis. *Curr. Opin. Plant Biol.* **11**:680–686.
- Melcher, U. 2000. The '30K' superfamily of viral movement proteins. *J. Gen. Virol.* **81**:257–266.
- Melcher, U., D. L. Steffens, D. J. Lyttle, G. Lebeurier, H. Lin, I. S. Choe, and R. C. Essenberg. 1986. Infectious and non-infectious mutants of cauliflower mosaic virus DNA. *J. Gen. Virol.* **67**:1491–1498.
- Perbal, M. C., C. L. Thomas, and A. J. Maule. 1993. Cauliflower mosaic virus gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. *Virology* **195**:281–285.
- Rojas, M. R., A. O. Noueiry, W. J. Lucas, and R. L. Gilbertson. 1998. Bean dwarf mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. *Cell* **95**:105–113.
- Sánchez-Navarro, J. A., and J. F. Bol. 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., M. C. Herranz, and V. Pallas. 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.
- Solovyev, A. G., D. A. Zelenina, E. I. Savenkov, V. Z. Grdzlishvili, S. Y. Morozov, D. E. Lesemann, E. Maiss, R. Casper, and J. G. Atabekov. 1996. Movement of a barley stripe mosaic virus chimera with a tobacco mosaic virus movement protein. *Virology* **217**:435–441.
- Stavolone, L., M. E. Villani, D. Leclerc, and T. Hohn. 2005. A coiled-coil interaction mediates cauliflower mosaic virus cell-to-cell movement. *Proc. Natl. Acad. Sci. U. S. A.* **102**:6219–6224.
- Tenllado, F., and J. F. Bol. 2000. Genetic dissection of the multiple functions of alfalfa mosaic virus coat protein in viral RNA replication, encapsidation, and movement. *Virology* **268**:29–40.
- Thomas, C. L., and A. J. Maule. 1999. Identification of inhibitory mutants of Cauliflower mosaic virus movement protein function after expression in insect cells. *J. Virol.* **73**:7886–7890.
- van der Kuyl, A. C., L. Neeleman, and J. F. Bol. 1991. Complementation and recombination between alfalfa mosaic virus RNA 3 mutants in tobacco plants. *Virology* **183**:731–738.