JOURNAL OF VIROLOGY, July 2006, p. 6712–6715 Vol. 80, No. 13
0022-538X/06/$08.00+0 doi:10.1128/JVI.00453-06

Challenging the Role of Microtubules in *Tobacco Mosaic Virus* Movement by Drug Treatments Is Disputable

Mark Seemanpillai, Rabab Elamawi, Christophe Ritzenthaler, and Manfred Heinlein*

Institut Biologie Moléculaire des Plantes, 12 Rue du Général Zimmer, 67084 Strasbourg Cedex, France

Received 3 March 2006/Accepted 10 April 2006

The movement protein (MP) of *Tobacco mosaic virus* interacts with microtubules during infection. Although this interaction is correlated with the function of MP in the cell-to-cell transport of viral RNA, a direct role of microtubules in the movement process was recently challenged by studies involving the treatment of plants with inhibitors of microtubule polymerization. Here, we report evidence suggesting that such treatments may not efficiently disrupt all microtubules. Thus, results obtained from studies using microtubule inhibitors may have to remain open to interpretation with regard to the involvement of microtubules in viral RNA trafficking.

Plant viruses have evolved specialized proteins termed "movement proteins" (MP) that govern intercellular spread of infection through plasmodesmata, cytoplasmic channels in the plant cell wall that connect adjacent cells. The 30-kDa protein of *Tobacco mosaic virus* (TMV) was the first MP identified. Similarly to many other MPs known today, this protein targets and modifies the size exclusion limit of plasmodesmata, spreads between cells if expressed in the absence of infection, and binds nucleic acids in vitro (reviewed in reference 12). Moreover, this protein has been shown to localize to the endoplasmic reticulum (ER) and to cytoskeletal elements (13, 14, 24) and to be phosphorylated by cellular kinases that may regulate its function (2, 8, 11, 16, 17, 20, 27, 28). However, despite these accumulated findings, the mechanism by which this protein facilitates the spread of infection is not yet well understood. In vivo assays using TMV derivatives expressing functional, dysfunctional, and temperature-sensitive mutants of MP fused to green fluorescent protein (GFP) provided evidence that the function of MP in viral RNA (vRNA) movement is strictly correlated with its ability to associate with microtubules (4–7, 19). A role of microtubules in movement is also suggested by the observed colocalization of vRNA with microtubules, which depends on microtubule-associated MP (22, 23). However, the potential role of microtubules was recently challenged by the observation that the spread of infection is not inhibited in leaves treated with inhibitors of the microtubule cytoskeleton (10, 18). Parallel treatments of *Nicotiana benthamiana* plants expressing *Arabidopsis* α-tubulin (TUA6) fused to GFP (*tua*-GFP) demonstrated the activity of the infiltrated inhibitor (10). However, it remained unclear whether the absence of *tua*-GFP-containing filaments also indicated the absence of microtubules made of endogenous tubulin. Here, we used *tua*-GFP-expressing BY-2 cells and *N. benthamiana* plants to test whether *tua*-GFP is a reliable marker for microtubules and whether microtubules are indeed efficiently disrupted upon infiltration of microtubule-disrupting agents.

* Corresponding author. Mailing address: Institut Biologie Moléculaire des Plantes, 12 Rue du Général Zimmer, 67084 Strasbourg Cedex, France. Phone: 33 3 88 41 72 58. Fax: 33 3 88 61 44 42. E-mail: manfred.heinlein@ibmp-ulp.u-strasbg.fr.

Since *tua*-GFP has to compete with endogenous α-tubulin for polymerization and since visualization of *tua*-GFP may depend largely on its local concentration, it is conceivable that this fluorescent marker may reveal some but not all microtubules in a given cell. To test this possibility, BY-2 suspension culture cells expressing *tua*-GFP were stained with an antibody against β-tubulin (Fig. 1). As expected, a fluorescent microtubule cytoskeleton attributable to *tua*-GFP was apparent in the cells (Fig. 1A). However, antibody staining revealed that not all microtubules had incorporated *tua*-GFP in amounts sufficient for detection by GFP fluorescence (Fig. 1B). To further test whether *tua*-GFP is a marker suitable for the detection of microtubules that may remain intact in the presence of microtubule polymerization inhibitors, we treated the cells for 3 h with 500 μM colchicine before staining them with antibody. As expected from previous studies applying 100 μM colchicine (10), this treatment resulted in a diffuse pattern of GFP signal (Fig. 1C). Interestingly, however, staining the cells with antibodies against β-tubulin revealed that a substantial microtubule cytoskeleton remained intact, regardless of prior exposure to colchicine (Fig. 1D). This finding clearly indicates that the information gained by using *tua*-GFP can be misleading with respect to the presence or absence of microtubules. Similar discrepancies between the existing microtubule pattern and the *tua*-GFP signal could be obtained by treatment of the cells with the inhibitors oryzalin and amiprophos-methyl (APM) (Fig. 2). The reason for the disparate patterns of *tua*-GFP and antibody labeling is unknown. It is possible that the GFP fusion, or the heterologous origin of the α-tubulin itself, rendered the fluorescent microtubules in the *tua*-GFP plants more sensitive to microtubule-destabilizing compounds. However, since disparate patterns can already be observed in nontreated cells, it appears more likely that the different patterns are simply due to differences in detection sensitivity. Whereas *tua*-GFP detection relies on a single fluorophore per *tua*-GFP molecule, the method of indirect antibody labeling amplifies the fluorescence signal due to the attachment of several fluorochromes to each antigen molecule.

Given the evidence that *tua*-GFP may not always be fully reliable as a marker for the detection of microtubules that may still be present following the application of microtubule-disrupting agents, we wondered whether published conditions for

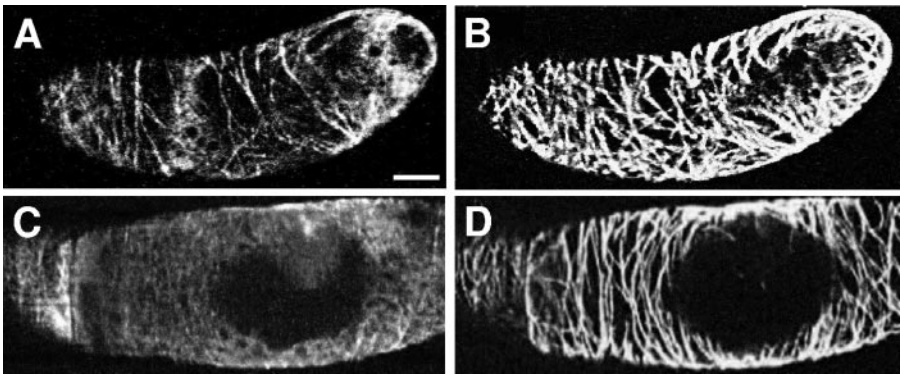

FIG. 1. Confocal fluorescence analysis of microtubule arrays in BY-2 cells expressing *Arabidopsis tua*-GFP. (A and B) *tua*-GFP incorporation allows visualization of only a subset of the total microtubule population in untreated cells. Bar = 10 μm. (A) *tua*-GFP-labeled microtubules. (B) Total microtubule population stained with anti-β-tubulin. (C and D) Colchicine treatment affects *tua*-GFP-labeled microtubules, although endogenous microtubules are still intact. (C) *tua*-GFP-labeled microtubules are no longer visualized following colchicine treatment. (D) Microtubules resistant to colchicine treatment and visualized by anti-β-tubulin staining.

microtubule disruption in epidermal cells verified by using *tua*-GFP as a marker indeed lead to the disruption of all microtubules in the treated cells. To test for the presence of such microtubules, we inoculated plants with a TMV that encodes a functional MP carrying a deletion of its 55 C-terminal amino acids, which are dispensable for function (MP^{C55}:GFP) (3, 7). However, compared to MP:GFP, the MP^{C55}:GFP derivative exhibits enhanced microtubule association in cells at the leading front of infection and, thus, is an ideal marker to reveal microtubules in newly infected cells. For the analysis of newly infected cells in tissues treated with microtubule polymerization inhibitors, we applied conditions as reported by Gillespie et al. (10) and Kawakami et al. (18). In short, leaf sections carrying infection sites were excised at 3 days postinoculation; syringe infiltrated with water, APM (50 μM), or colchicine (100 μM); maintained on the infiltration medium for 1 day; and finally analyzed by fluorescence microscopy. As shown in Fig. 3, infection by MP^{C55}:GFP continued to spread despite the presence of either colchicine or APM, as expected. Moreover, the binding of MP^{C55}:GFP to microtubules revealed that in control infection sites infiltrated with water (Fig. 3A and B), the microtubule array was unaffected (Fig. 3C) but that the microtubule cytoskeleton in tissues treated with either colchicine (Fig. 3D and E) or APM (Fig. 3G and H) was largely disrupted. In the absence of an intact microtubule cytoskeleton, MP^{C55}:GFP showed a range of localization patterns. Importantly, these patterns also included various patterns of microtubules ranging from short filaments (Fig. 3F) to seemingly intact arrays (Fig. 3I). The observation that MP^{C55}:GFP labels microtubules in newly infected cells treated with microtubule inhibitors indicates that the infiltrated inhibitors do not cause the full disruption of all microtubules. Thus, while it seems clear that the spread of TMV infection does not require an intact microtubule cytoskeleton, the possibility of a role for individual microtubule activities in the movement process cannot be excluded.

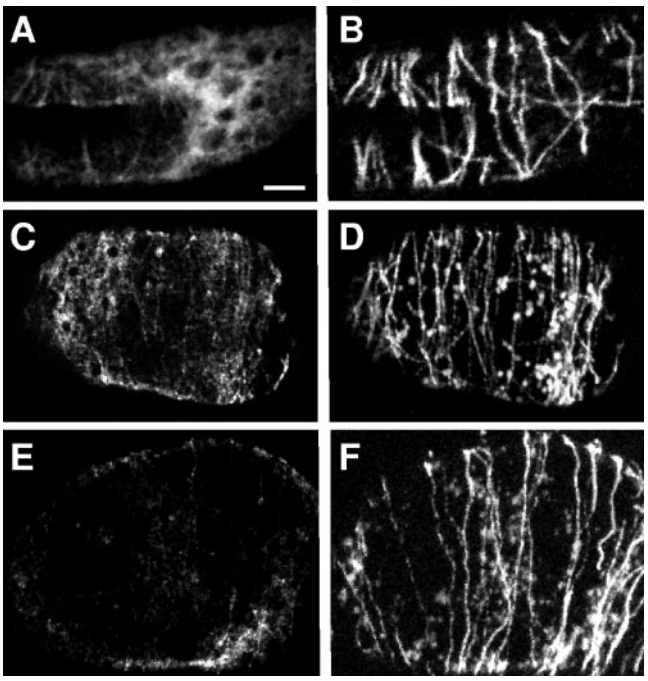

FIG. 2. Confocal fluorescence analysis of microtubule arrays in BY-2 cells treated with APM and oryzalin. (A to D) Cells treated with 50 μM APM for 15 min. APM treatment causes diffuse *tua*-GFP fluorescence, although microtubules are still present. Bar = 10 μm. (A) Diffuse *tua*-GFP fluorescence. (B) Microtubules stained with anti-β-tubulin. (C) Diffuse *tua*-GFP fluorescence and some weakly labeled filaments. (D) Microtubules stained with anti-β-tubulin. (E and F) Cells treated with 10 μM oryzalin for 40 min. (E) Absence of *tua*-GFP-labeled filaments. (F) Presence of microtubules stained by anti-β-tubulin.

Previous studies applying microtubule inhibitors indicated that microtubules are not required for the cell-to-cell movement of TMV infection (10, 18). However, doubt remained since these studies did not conclusively demonstrate that indeed all microtubules are disrupted following the treatments. Although *tua*-GFP was used as a marker for microtubule disruption (10), it remained possible that this marker may not be sufficiently sensitive for revealing microtubules that may have remained intact following the treatments. Our observations reported here indicate that *tua*-GFP indeed does not always properly reveal the presence of microtubules. We also show

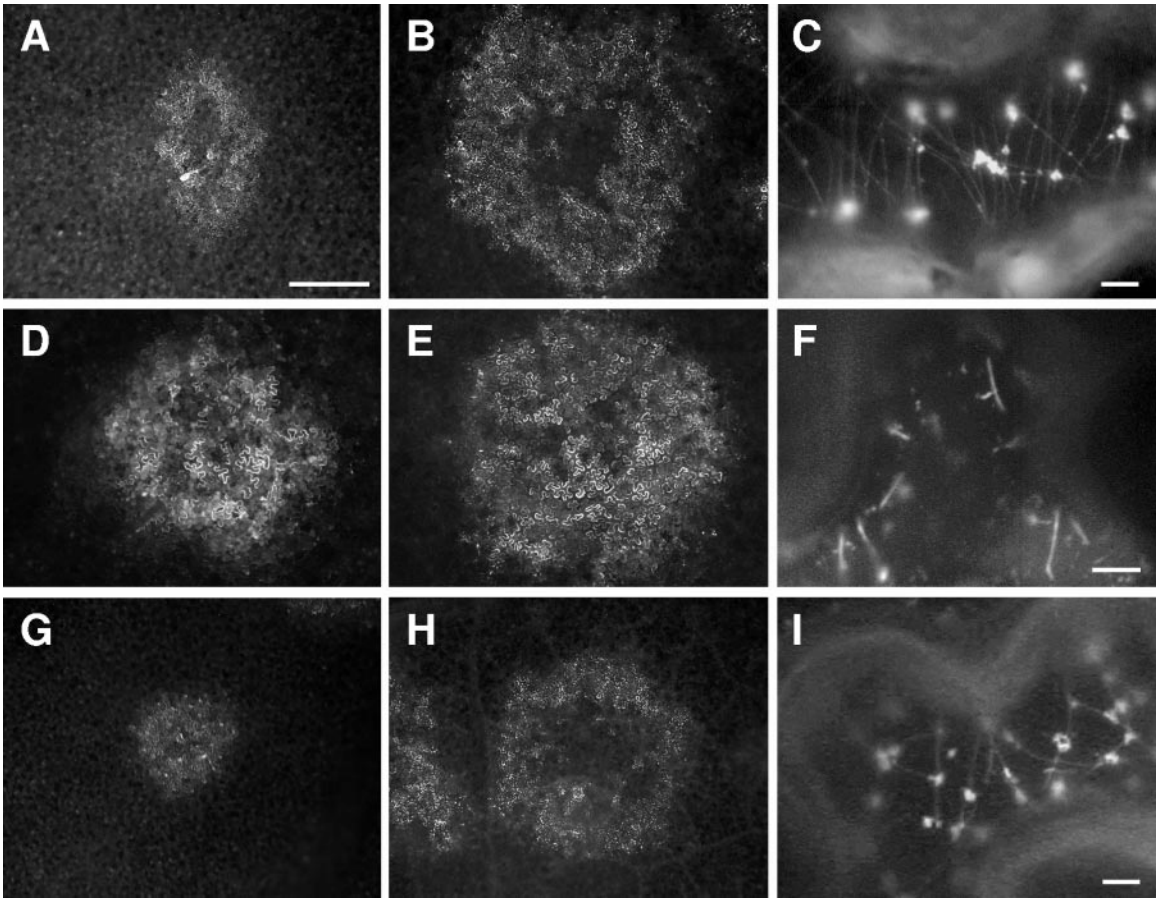

FIG. 3. Spread of TMV-MP^{C55}:GFP infection and presence of microtubules in cells treated with microtubule polymerization inhibitors. (A to C) Infection site infiltrated with water. (A) Before treatment. Bar = 1 mm (applies also to panels B, D, E, G, and H). (B) Twenty-four hours after treatment. (C) Cell at the leading front of infection showing the association of MP^{C55}:GFP with microtubules and bodies (ER aggregates). Bar = 5 μm. (D to F) Infection site infiltrated with 100 μM colchicine. (D) Before treatment. (E) Twenty-four hours after treatment. (F) Cell at the leading front of infection showing the association of MP^{C55}:GFP with short microtubules. Bar = 5 μm. (G to I) Infection site infiltrated with 50 μM APM. (G) Before treatment. (H) Twenty-four hours after treatment. (I) Cell at the leading front of infection showing the association of MP^{C55}:GFP with microtubules and bodies. Bar = 5 μm.

evidence that the treatment of leaf tissues with microtubule-disrupting agents does not lead to the disruption of all microtubules. Given this evidence indicating that specific treatments with microtubule polymerization inhibitors may not disrupt all microtubules and that the *tua*-GFP-fluorescent microtubules may not accurately represent the microtubule population as a whole, we suggest that results obtained from studies utilizing *tua*-GFP as a marker to indicate microtubule disruption should remain open to interpretation with regard to the involvement of microtubules in TMV cell-to-cell trafficking.

The possibility that the inhibitors may not be fully effective must be taken very seriously in this case since the spread of infection requires the movement of only very few virus genomes (21, 26). Moreover, since TMV establishes several ER-associated infection sites within infected leaf cells (14, 25) and since many of these infection sites are located in direct vicinity to plasmodesmata (25), localized processes in plasmodesma-proximal sites may suffice to ensure vRNA movement and the spread of infection into adjacent cells. Given that (i) both the MP and the viral genome are expressed to high levels during infection (1, 25), that (ii) virus movement requires very few virus particles (21, 26), and that (iii) local events at one of the many plasmodesmata that connect a cell with adjacent cells may suffice for virus movement, the inhibition of virus movement may be extremely difficult to achieve unless a full disruption of the transport mechanism can be established. Since MP binds microtubules in various systems, including mammalian cells (5, 9) and procaryotes (15), as well as in vitro (24) (J. Ashby et al., unpublished data), and since the ability of MP to associate with microtubules has been functionally correlated with virus movement in vivo (4–7) and with the presence of microtubule-associated vRNA (22, 23), a potential role of microtubule-based activities in TMV movement should be further investigated. If future studies demonstrate that microtubules indeed have no direct role in viral RNA movement, it will remain to be asked why this interaction is conserved in various cell types and why it correlates with MP function.

This work was performed with financial support from the CNRS and the French Ministry of Research (ACI-BCMS187). M.S. was supported by a CNRS postdoctoral fellowship, and R.E. received financial support from the Republic of Egypt.

We thank K. Oparka for providing seeds of the *tua*-GFP-expressing *N. benthamiana* line.

REFERENCES

1. **Arce-Johnson, P., T. W. Kahn, U. Reimann-Philipp, R. Rivera-Bustamente, and R. N. Beachy.** 1995. The amount of movement protein produced in transgenic plants influences the establishment, local movement, and systemic spread of infection by movement protein-deficient tobacco mosaic virus. Mol. Plant-Microbe Interact. **3:**415–423.
2. **Atkins, D., R. Hull, B. Wells, K. Roberts, P. Moore, and R. N. Beachy.** 1991. The tobacco mosaic virus 30K movement protein in transgenic tobacco plants is localized to plasmodesmata. J. Gen. Virol. **72:**209–211.
3. **Berna, A., R. Gafny, S. Wolf, W. J. Lucas, C. A. Holt, and R. N. Beachy.** 1991. The TMV movement protein: role of the C-terminal 73 amino acids in subcellular localization and function. Virology **182:**682–689.
4. **Boyko, V., J. A. Ashby, E. Suslova, J. Ferralli, O. Sterthaus, C. M. Deom, and M. Heinlein.** 2002. Intramolecular complementing mutations in *Tobacco mosaic virus* movement protein confirm a role for microtubule association in viral RNA transport. J. Virol. **76:**3974–3980.
5. **Boyko, V., J. Ferralli, J. Ashby, P. Schellenbaum, and M. Heinlein.** 2000. Function of microtubules in intercellular transport of plant virus RNA. Nat. Cell Biol. **2:**826–832.
6. **Boyko, V., J. Ferralli, and M. Heinlein.** 2000. Cell-to-cell movement of TMV RNA is temperature-dependent and corresponds to the association of movement protein with microtubules. Plant J. **22:**315–325.
7. **Boyko, V., J. van der Laak, J. Ferralli, E. Suslova, M.-O. Kwon, and M. Heinlein.** 2000. Cellular targets of functional and dysfunctional mutants of tobacco mosaic virus movement protein fused to green fluorescent protein. J. Virol. **74:**11339–11346.
8. **Citovsky, V., B. G. McLean, J. R. Zupan, and P. Zambryski.** 1993. Phosphorylation of tobacco mosaic virus cell-to-cell movement protein by a developmentally regulated plant cell wall-associated protein kinase. Genes Dev. **7:**904–910.
9. **Ferralli, J., J. Ashby, M. Fasler, V. Boyko, and M. Heinlein.** 2006 Disruption of microtubule organization and centrosome function by expression of *Tobacco mosaic virus* movement protein. J. Virol. **80:**5807–5821.
10. **Gillespie, T., P. Boevink, S. Haupt, A. G. Roberts, R. Toth, T. Vantine, S. Chapman, and K. J. Oparka.** 2002. Functional analysis of a DNA shuffled movement protein reveals that microtubules are dispensable for the cell-to-cell movement of tobacco mosaic virus. Plant Cell **14:**1207–1222.
11. **Haley, A., T. Hunter, P. Kiberstis, and D. Zimmern.** 1995. Multiple serine phosphorylation sites on the 30 kDa TMV cell-to-cell movement protein synthesized in tobacco protoplasts. Plant J. **8:**715–724.
12. **Heinlein, M., and B. L. Epel.** 2004. Macromolecular transport and signaling through plasmodesmata. Int. Rev. Cytol. **235:**93–164.
13. **Heinlein, M., B. L. Epel, H. S. Padgett, and R. N. Beachy.** 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science **270:**1983–1985.
14. **Heinlein, M., H. S. Padgett, J. S. Gens, B. G. Pickard, S. J. Casper, B. L. Epel, and R. N. Beachy.** 1998. Changing patterns of localization of the tobacco mosaic virus movement protein and replicase to the endoplasmic reticulum and microtubules during infection. Plant Cell **10:**1107–1120.
15. **Heinlein, M., M. R. Wood, T. Thiel, and R. N. Beachy.** 1998. Targeting and modification of prokaryotic cell-cell junctions by tobacco mosaic virus cell-to-cell movement protein. Plant J. **14:**345–351.
16. **Kawakami, S., K. Hori, D. Hosokawa, Y. Okada, and Y. Watanabe.** 2003. Defective tobamovirus movement protein lacking wild-type phosphorylation sites can be complemented by substitutions found in revertants. J. Virol. **77:**1452–1461.
17. **Kawakami, S., H. S. Padgett, D. Hosokawa, Y. Okada, R. N. Beachy, and Y. Watanabe.** 1999. Phosphorylation and/or presence of serine 37 in the movement protein of tomato mosaic tobamovirus is essential for intracellular localization and stability in vivo. J. Virol. **73:**6831–6840.
18. **Kawakami, S., Y. Watanabe, and R. N. Beachy.** 2004. Tobacco mosaic virus infection spreads cell to cell as intact replication complexes. Proc. Natl. Acad. Sci. USA **101:**6291–6296.
19. **Kotlizky, G., A. Katz, J. van der Laak, V. Boyko, M. Lapidot, R. N. Beachy, M. Heinlein, and B. L. Epel.** 2001. A dysfunctional movement protein of *Tobacco mosaic virus* interferes with targeting of wild type movement protein to microtubules. Mol. Plant-Microbe Interact. **7:**895–904.
20. **Lee, J. Y., K. Taoka, B. C. Yoo, G. Ben-Nissan, D. J. Kim, and W. J. Lucas.** 2005. Plasmodesmal-associated protein kinase in tobacco and Arabidopsis recognizes a subset of non-cell-autonomous proteins. Plant Cell **17:**2817–2831.
21. **Li, H., and M. J. Roossinck.** 2004. Genetic bottlenecks reduce population variation in an experimental RNA virus population. J. Virol. **78:**10582–10587.
22. **Más, P., and R. N. Beachy.** 1999. Replication of tobacco mosaic virus on endoplasmic reticulum and role of the cytoskeleton and virus movement in intracellular distribution of viral RNA. J. Cell Biol. **147:**945–958.
23. **Más, P., and R. N. Beachy.** 2000. Role of microtubules in the intracellular distribution of tobacco mosaic virus movement protein. Proc. Natl. Acad. Sci. USA **97:**12345–12349.
24. **McLean, B. G., J. Zupan, and P. C. Zambryski.** 1995. Tobacco mosaic virus movement protein associates with the cytoskeleton in tobacco plants. Plant Cell **7:**2101–2114.
25. **Padgett, H. S., B. L. Epel, T. W. Kahn, M. Heinlein, Y. Watanabe, and R. N. Beachy.** 1996. Distribution of tobamovirus movement protein in infected cells and implications for cell-to-cell spread of infection. Plant J. **10:**1079–1088.
26. **Sacristán, S., J. M. Malpica, A. Fraile, and F. García-Arenal.** 2003. Estimation of population bottlenecks during systemic movement of tobacco mosaic virus in tobacco plants. J. Virol. **77:**9906–9911.
27. **Waigmann, E., M.-H. Chen, R. Bachmeier, S. Ghoshroy, and V. Citovsky.** 2000. Regulation of plasmodesmal transport by phosphorylation of tobacco mosaic virus cell-to-cell movement protein. EMBO J. **19:**4875–4884.
28. **Watanabe, Y., T. Meshi, and Y. Okada.** 1992. *In vivo* phosphorylation of the 30-kDa protein of tobacco mosaic virus. FEBS Lett. **313:**181–184.