Research Note

Effects of Movement Protein Mutations on the Formation of Tubules in Plant Protoplasts Expressing a Fusion Between the Green Fluorescent Protein and *Cauliflower mosaic virus* Movement Protein

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Fusions between the green fluorescent protein (GFP) and the *Cauliflower mosaic virus* (CaMV) movement protein (MP) induce the formation of fluorescent foci and surface tubules in *Arabidopsis thaliana* leaf mesophyll protoplasts. Tubules elongate coordinately and progressively in an assembly process approximately 6 to 12 h following transfection of protoplasts with GFP-MP constructs. Tubules are not formed in protoplasts transfected by GFP-MP^{ER2A}, a MP mutation that renders CaMV noninfectious. A small number of short tubules are formed on protoplasts transfected by GFP-MP^{N6} and GFP-MP^{N13}, two second-site revertants of ER2A that partially restore infectivity. Protoplasts cotransfected with cyan fluorescent protein (CFP)-MP^{WT} and GFP-MP^{ER2A} form tubules containing both MP fusions, indicating that although the GFP-MP^{ER2A} cannot induce tubule formation, GFP-MP^{ER2A} can coassemble or colocalize with CFP-MP^{WT} in tubules. Thus, CaMV MP-induced tubule formation in protoplasts correlates closely with the infectivity of mutation ER2A and its revertants, suggesting that tubule-forming capacity in plant protoplasts reflects a process required for virus infection or movement.

Additional keywords: epifluorescence, plasmodesmata, virus movement, Western blot.

During systemic infections in plants, viruses or viral RNA-protein complexes move locally from cell to cell as well as long distance through the vasculature (Carrington et al. 1996; Lazarowitz and Beachy 1999). Virus movement is potentiated by virally encoded movement proteins (MPs), which are often encoded by a single gene, as in tobamoviruses, or as a triple block of genes, as in potex-, carla-, hordei-, and furoviruses. A number of plant viruses, typified by *Tobacco mosaic virus*

(TMV), do not require a viral coat protein for movement and their MPs bind RNA (Citovsky et al. 1990; Citovsky et al. 1992), suggesting that viral MPs may mediate viral transport as an RNA-protein complex (Citovsky and Zambryski 1991; Citovsky and Zambryski 1993; McLean et al. 1993). TMV MP localizes to a number of different subcellular locations (Kahn et al. 1998; Mas and Beachy 1998; Mas and Beachy 1999; Mas and Beachy 2000), including plasmodesmata that may be modified, thereby increasing their size exclusion limit (Olesinski et al. 1996; Oparka et al. 1997; Waigmann et al. 1994; Wolf et al. 1989).

Other plant viruses such as comoviruses require a coat protein for movement and their MPs induce the formation of tubular extensions in infected cells (Kasteel et al. 1996; Van Lent et al. 1991). In *Cowpea mosaic virus* (CPMV), the viral MP is in association with the tubular structures (Kasteel et al. 1997; Van Lent et al. 1990). Virus particles also have been observed in tubules, suggesting that virus particles move through tubular extensions from cell to cell (Van Lent et al. 1990; Van Lent et al. 1991).

In caulimoviruses such as *Cauliflower mosaic virus* (CaMV, a member of the family *Caulimoviridae*), it is not clear whether the virus moves from cell to cell as a virion or a viral RNA-protein complex (Carrington et al. 1996). The CaMV MP binds single-strand nucleic acids (Citovsky et al. 1991; Thomas and Maule 1995), and it has been proposed that a RNA-protein complex involving the 35S RNA is transported from cell to cell (Thomas and Maule 1995). CaMV or the CaMV MP (gene I product) expressed from a plasmid vector, however, induces tubule formation in protoplasts (Kasteel et al. 1996; Perbal et al. 1993), and caulimovirus particles have been observed in the plasmodesmata of infected cells (Linstead et al. 1988).

Recently, we reported that constructs encoding green fluorescent protein (GFP)-CaMV MP fusions are able to induce the formation of fluorescent foci and surface tubules when transfected into *Arabidopsis thaliana* protoplasts (Huang et al. 2000). In this study, we use GFP-MP fusions to study the effects of MP mutations on tubule formation. To do so, *A. thaliana* mesophyll protoplasts were transfected with GFP-MP

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fusion constructs and monitored by fluorescent microscopy through a time course of 24 h. *Arabidopsis mesophyll* protoplasts were isolated and transfected, as described by Abel and Theologis (1994). Protoplasts (1×10^6) were mixed with 40 μ g of plasmid DNA (20 μ g of each plasmid in cotransfection experiments) and then treated with 40% polyethylene glycol solution for 30 min. After washing, protoplasts were resuspended in 1.5 ml of modified MS medium (Murashige and Skoog 1962) with B5 vitamins plus 0.5 M mannitol and incubated in the dark at 21°C.

Because we were unable to immobilize and follow single protoplasts, the appearance of fluorescent cells, foci, and tubules in a population of protoplasts was monitored. Dimly fluorescent cells were first observed approximately 4 h posttransfection. By 6 h posttransfection, brightly fluorescent cells and some cells with fluorescent foci or newly emerging tubules were observed (Fig. 1A). Tubules appeared to elongate coordinately on any single cell and progressively in the protoplast population, reaching maximum numbers and length in approximately 12 h (Fig. 1B to D). As we reported previously, approximately 20 to 30% of fluorescent protoplasts formed tubules and fluorescent foci and another 20 to 30% only formed fluorescent foci.

In another study, we described the generation of site-directed MP mutants used to search for host suppressors of MP mutations (Callaway et al. 2000). One of the mutants utilized in that analysis was ER2A, a two-codon mutation with a F deletion at position 73 and a R to A substitution at position 75. ER2A is noninfectious, although revertants N6 and N13 were picked up as second-site MP mutations in a large population of ER2A-inoculated plants (Callaway et al. 2000). N6 and

N13 revertants retained the original ER2A mutation, although N6 also contained a V to F substitution at position 68 and N13 had an E to K substitution at position 78. The second-site mutations partially restored infectivity in that revertants were infectious but produced symptoms more slowly (Callaway et al. 2000), accumulating to lower titers than their wild-type counterpart (A. S. Callaway, *unpublished observations*). Compared to the wild-type virus, symptoms produced in the N13 revertant were delayed by 5 days and, in the N6 revertant, symptoms were delayed by 4 days (Callaway et al. 2000).

We investigated whether the ER2A mutation and the second-site revertants would affect tubule formation in *A. thaliana* protoplasts. GFP-MP mutant fusion constructs were generated by replacing the corresponding MP gene with amplified fragments from MP mutants, resulting in the plasmids pIBT-GFP-MP^{ER2A}, pIBT-GFP-MP^{N6}, and pIBT-GFP-MP^{N13}. Protoplasts transfected with constructs containing the ER2A mutation (pIBT-GFP-MP^{ER2A}) failed to induce tubules after 24 h posttransfection, although more protoplasts produced fluorescent foci (Fig. 2B and Table 1). The larger percentage of protoplasts with only fluorescent foci (46% for GFP-MP^{ER2A} compared with 24% for GFP-MP^{WT}) appeared to derive from the population of protoplasts failing to form tubules. Protoplasts transfected with constructs bearing the N6 or N13 reversion (GFP-MP^{N6} and GFP-MP^{N13}, respectively) produced tubules, although a smaller percentage of protoplasts formed tubules that were considerably shorter and fewer per protoplast (Fig. 2C and D and Table I). Although fewer tubules were formed in protoplasts transformed by the N6 and N13 revertants, they were quite different from protoplasts transfected with the ER2A mutation, which formed no tubules at all. Hence, the capacity of the mutants to form tubules generally correlated with the infectivity of the virus. The ER2A

Fig. 1. Time course of tubule formation in *Arabidopsis thaliana* protoplasts. Protoplasts were transfected with GFP-MP^{WT} constructs and observed at various times posttransfection with a BX50 epifluorescence microscope (Olympus, Melville, NY, U.S.A.) equipped with a CCD camera (Princeton Scientific Instruments, Monmouth Junction, NJ, U.S.A.). Protoplasts observed at **A**, 6 h; **B**, 8 h; **C**, 10 h; and **D**, 12 h posttransfection. Digital images were processed with Photoshop software (Adobe Systems, San Jose, CA, U.S.A.). Bar = 20 μ m.

Fig. 2. Tubule formation in *Arabidopsis thaliana* protoplasts by movement protein (MP) mutants. Protoplasts were transfected with various green fluorescent protein (GFP)-MP mutant constructs and observed 24 h later. Protoplasts transfected with **A**, GFP-MP^{WT}; **B**, GFP-MP^{ER2A}; **C**, GFP-MP^{N6}; and **D**, GFP-MP^{N13}. Bar = 20 μ m.

mutation is not infectious, and no tubules were formed. Infectivity was restored partially in the N6 and N13 revertants, and protoplasts transfected with the revertants formed tubules, albeit ones that were shorter and fewer in number. The inability of the ER2A mutant to form tubules was not the result of a corresponding failure to accumulate the mutant protein. On Western blots, we found comparable-to-slightly less GFP-MP^{ER2A}, GFP-MP^{N6}, and GFP-MP^{N13} (Fig. 3C to E) in extracts from protoplasts harvested at 24 h posttransfection compared with GFP-MP^{WT} (Fig. 3B), a finding that was reinforced by visual observations in the microscope in which protoplasts transfected with the different GFP-MP constructs generally fluoresced with approximately the same intensities.

Although GFP-MP^{ER2A} failed to induce tubules, we investigated whether the mutant interfered with tubule formation induced by GFP-MP^{WT} in protoplasts cotransfected with the two constructs. To monitor both MPs, double-labeling experiments were carried out with different fluorescent protein fusions and cotransfecting *A. thaliana* protoplasts with equal amounts of constructs encoding cyan fluorescent protein (CFP)-MP^{WT} and GFP-MP^{ER2A}. Fluorescent protein genes were amplified by polymerase chain reaction from plasmids pECFP and pEYFP-Nuc (6075-1 and 6905-1, respectively; Clontech, Palo Alto CA, U.S.A.) and used to replace the corresponding GFP gene in plasmids pIBT-GFP and pIBT-GFP-MP, described previously (Huang et al. 2000), giving rise to plasmids pIBT-CFP, pIBT-CFP-MP, pIBT-yellow fluorescent protein (YFP), and pIBT-YFP-MP, respectively.

In control experiments, CFP-MP^{WT} induced the formation of tubules, and CFP fluorescence was visible only through a CFP filter (Fig. 4A and B). When CFP-MP^{WT} was cotransfected with GFP or YFP (although not as a MP fusion), protoplasts and tubules were fluorescent when viewed through a CFP filter, but only the “cell body” fluoresced through the YFP filter (Fig. 4C to F). This control demonstrates that free GFP and YFP are not in tubules at levels that can be detected by fluorescence microscopy. Additional control experiments with protoplasts cotransfected with CFP-MP^{WT} and YFP-MP^{WT} or CFP-MP^{WT} and GFP-MP^{WT} demonstrate that protoplasts with tubules were double labeled, i.e., protoplasts and tubules fluoresced in CFP and YFP channels (Fig. 4G to J).

Protoplasts cotransfected with equal quantities of CFP-MP^{WT} and GFP-MP^{ER2A} constructs form comparable numbers of tubules relative to protoplasts transfected with GFP-MP^{WT} (Fig. 4K and L). Therefore, at these levels of GFP-MP^{ER2A} expression, the mutant does not interfere with the action of CFP-MP^{WT}. We have not tested whether GFP-MP^{ER2A}, if highly overexpressed relative to CFP-MP^{WT}, would interfere with tubule formation. Fluorescence in tubules was observed through the CFP and YFP filters, indicating that GFP-MP^{ER2A} is in tubules induced by CFP-MP^{WT}. Similar observations

were made with the revertants N6 and N13. Recall that GFP-MP^{N6} and GFP-MP^{N13} induce very few, short tubules, and that GFP-MP^{N6} and GFP-MP^{N13} were in tubules of the number and length induced by CFP-MP^{WT} (Fig. 4M to P; note that the ends of several tubules in these images are out of the plane of focus.) We interpret this to mean that although the GFP-MP^{ER2A} is incapable of tubule formation and GFP-MP^{N6} and GFP-MP^{N13} are only modestly effective in inducing tubule formation in transfected protoplasts, they are, nonetheless, capable of assembling or localizing in tubules induced by the wild-type MP.

It has been suggested by Thomas and Maule (2000) that the CaMV MP may organize or assemble to form structures that give rise to surface tubules. Thus, they created N-terminal MP-GFP and C-terminal GFP-MP fusions and tested them for tubule formation in cultured insect cells. They did not observe fluorescent tubule formation when insect cells were infected with recombinant Baculovirus encoding these fusions. They did, however, observe tubules when cells were coinfecting with the MP fusions and an unfused MP construct. They also observed tubules when cells were infected with a construct containing an autoproteolytic peptide cleavage site between the MP and GFP moieties (the peptide cleavage action produced a mixed population of cleaved and uncleaved fusion proteins). They interpreted this to mean that the GFP moiety in the MP fusions probably interferes with the productive assembly of MP that leads to tubule formation. They did find, however, that the MP fusions can participate in assemblies created by unfused MP.

In our previous experiments, C-terminal-GFP-MP fusions were active in producing tubules in plant cells, whereas N-terminal-MP-GFP fusions were not (Huang et al. 2000). We do not know why our C-terminal-GFP-MP fusions are active in tubule formation in plant protoplasts and those generated by Thomas and Maule (2000) were not, although subtle differences in the constructs may explain the results. Because our GFP-MP fusions are active in tubule formation, this should not be interpreted to mean that the CaMV MP does not self-assemble or organize in promoting tubule formation. It may simply mean that the C-terminal fusion does not interfere with an assembly or interaction process required for tubule growth. We do not know whether the fusion proteins actually are active in virus movement.

Fig. 3. Western blot of green fluorescent protein (GFP) movement protein (MP) expression in transfected protoplasts. Protoplasts were transfected with various GFP-MP mutant constructs: **A**, GFP; **B**, GFP-MP^{WT}; **C**, GFP-MP^{ER2A}; **D**, GFP-MP^{N6}; and **E**, GFP-MP^{N13}. Protein samples from 1×10^6 protoplasts harvested 24 h posttransfection were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli 1970) and transferred to polyvinylidene difluoride membranes, as described (Sambrook et al. 1989). The membranes were probed with an anti-MP antibody against the *Cauliflower mosaic virus* MP (Maule et al. 1992), followed by a horseradish peroxidase-conjugated secondary antibody (Promega, Madison WI, U.S.A.).

Table 1. Effect of MP mutants on tubule formation

Construct	Protoplasts w/tubules and fluorescent foci	Protoplasts w/fluorescent foci	Total
GFP-MP ^{WT}	22 (29%)	18 (24%)	75
GFP-MP ^{ER2A}	0 (0%)	32 (46%)	69
GFP-MP ^{N6}	5 ^a (7%)	28 (41%)	68
GFP-MP ^{N13}	4 ^a (6%)	32 (46%)	70

^a One to three short tubules per protoplast.

It is still unresolved whether the formation of surface tubules represents some function related to virus movement and to the normal biological activity of the MP. In our study of the ER2A MP mutation and its revertants, the capacity of MP

mutants to form surface tubules in protoplasts correlated with their infectivity in plants. The ER2A mutant is not infectious in *A. thaliana* (Col-0 ecotype), and the same mutation in a GFP-MP fusion fails to induce tubules in protoplasts. Two

Fig. 4. Tubule formation in protoplasts cotransfected with different fluorescent protein movement protein (MP) constructs. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) genes were amplified by polymerase chain reaction from plasmids pECFP and pEYFP-Nuc (6075-1 and 6905-1, respectively; Clontech, Palo Alto, CA, U.S.A.) and used to replace the corresponding green fluorescent protein (GFP) gene in plasmids pIBT-GFP and pIBT-GFP-MP or in the MP mutant derivatives (Huang et al. 2000). Protoplasts were cotransfected with various fusion protein constructs and observed 24 h later. **A and B**, CFP-MP^{WT}. **C and D**, CFP-MP^{WT} + GFP. **E and F**, CFP-MP^{WT} + YFP. **G and H**, CFP-MP^{WT} + YFP-MP^{WT}. **I and J**, CFP-MP^{WT} + GFP-MP^{WT}. **K and L**, CFP-MP^{WT} + GFP-MP^{ER2A}. **M and N**, CFP-MP^{WT} + GFP-MP^{N6}. **O and P**, CFP-MP^{WT} + GFP-MP^{N13}. Chroma Technologies filter sets (Brattleboro, VT, U.S.A.) were used and included a GFP filter (41017; 470–40-nm excitation with 525–50-nm emission), CFP filter (31044v2; 436–20-nm excitation with 480–40-nm emission), and YFP filter (41028; 500–20-nm excitation with 535–30-nm emission). In cotransfection experiments, transfected protoplasts were viewed successively and photographed with the CFP and YFP filters. **A, C, E, G, I, K, M, O**, Photographs through the CFP filter. **B, D, F, H, J, L, N, and P**, Photographs through the YFP filter. Bar = 20 μ m.

revertants (second-site MP mutations) of ER2A that partially restore infectivity form a small number of short-surface tubules in protoplasts, although the viruses show a delay in the appearance of symptoms (Callaway et al. 2000). The ER2A and second-site mutations are located in the N-terminal region of the MP within a domain we identified through MP-deletion analysis, which is required for tubule formation, but outside of a domain required for fluorescent focus formation in protoplasts (Huang et al. 2000). The effects of the ER2A and second-site mutations in this study are consistent with the CaMV MP domain map (Huang et al. 2000) in that the mutations affect tubule but not fluorescent-focus formation.

Canto and Palukaitis (1999) argued through the analysis of MP mutations in CMV that the capacity to induce surface tubules in protoplasts is unrelated to the ability of the virus to move systemically in infected plants. They found that the M8 mutation in RNA 3A, the CMV MP, did not affect the spread of CMV in *Nicotiana tabacum* and *Nicotiana benthamiana*, even though it did impair the ability of M8 to induce tubules in protoplasts from these species. It is difficult to interpret the role of surface tubules in protoplasts expressing the CMV MP, however, because tubules have not been observed in CMV-infected plants and it is not considered likely that CMV moves by a tubule-mediated mechanism. Nonetheless, in light of these issues with CMV, it is important to examine CaMV for the effects of other mutations to see whether the correlation between the capacity to form tubules and infectivity holds.

In CaMV, the formation of surface tubules in GFP-MP-transfected protoplasts appears to result from an assembly process that may result from the interaction of MPs with each other or with host components. We used the yeast two-hybrid system to identify *Arabidopsis* proteins with which the CaMV MP interacts (Z. Huang, V. Andrianov, Y. Han, and S. Howell, unpublished). The interactions with one of the proteins, MPI7, are sensitive to the same mutations described in this study. That is, MP-MPI7 interactions are disrupted by the ER2A mutation and restored partially by the N6 and N13 revertants. Curiously, MPI7-GFP is not located in tubules but in cytoplasmic fluorescent foci-punctate spots from which tubules appear to emanate.

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