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Intracellular Localization and Movement Phenotypes of *Alfalfa mosaic virus* Movement Protein Mutants

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Thirteen mutations were introduced in the movement protein (MP) gene of *Alfalfa mosaic virus* (AMV) fused to the green fluorescent protein (GFP) gene and the mutant MP–GFP fusions were expressed transiently in tobacco protoplasts, tobacco suspension cells, and epidermal cells of tobacco leaves. In addition, the mutations were introduced in the MP gene of AMV RNA 3 and the mutant RNAs were used to infect tobacco plants. Ten mutants were affected in one or more of the following functions of MP: the formation of tubular structures on the surface of protoplasts, association with the endoplasmic reticulum (ER) of suspension cells and epidermal cells, targeting to punctate structures in the cell wall of epidermis cells, movement from transfected cells to adjacent cells in epidermis tissue, cell-to-cell movement, or long-distance movement in plants. The mutations point to functional domains of the MP and support the proposed order of events in AMV transport. Studies with several inhibitors indicate that actin or microtubule components of the cytoskeleton are not involved in tubule formation by AMV MP. Evidence was obtained that tubular structures on the surface of transfected protoplasts contain ER- or plasmalemma-derived material.

Cell-to-cell transport of plant viruses is mediated by one or more movement proteins (MPs) encoded by the viral genome. In addition, these MPs are involved in long-distance transport of the virus through the vascular system of the plant (Carrington et al. 1996; Crawford and Zambryski 1999; Lazarowitz and Beachy 1999; Lucas 1995; Lucas and Gilbertson 1994; Mezitt and Lucas 1996; Santa Cruz 1998). Two mechanisms of viral cell-to-cell transport have been distinguished (Carrington et al. 1996). In one model, exemplified by *Tobacco mosaic virus* (TMV), the virus moves from cell to cell through modified plasmodesmata as a ribonucleoprotein complex. The viral coat protein (CP) is not required, and the inner structure of the plasmodesmata is maintained. In the second model, exemplified by *Cowpea mosaic virus* (CPMV), viral movement does require CP, and the virus moves from cell to cell in the form of virions or encapsidated RNA through large tubular structures that traverse the plasmodesmata, thereby destroying the plasmodesmal inner structure.

Currently, it is not clear which mechanism of cell-to-cell transport is used by *Alfalfa mosaic virus* (AMV, family *Bromoviridae*). AMV is a positive-strand RNA virus with a tripartite genome. RNAs 1 and 2 are monocistronic and encode proteins P_1 and P_2, which are part of the viral replicase. RNA 3 is bicistronic and encodes the MP (32 kDa) and CP (24 kDa), which is translated from a subgenomic messenger, RNA 4. The MP contains RNA binding activity (Schoumacher et al. 1994) and can induce a limited increase of the size-exclusion limit of plasmodesmata (Poirson et al. 1993). MP and CP were required for cell-to-cell transport, but movement of CP mutants that did not form detectable virus particles was observed (Van der Vossen et al. 1994; Van der Vossen et al. 1995).

Transient expression of MP in tobacco protoplasts resulted in the formation of tubular structures on the protoplast surface (Zheng et al. 1997). When protoplasts were infected with AMV, these tubules appeared to be filled with virus particles (Kasteel et al. 1997). From a deletion analysis of MP–green fluorescent protein (GFP) fusion proteins expressed transiently from a plasmid (Zheng et al. 1997) or expressed from AMV RNA 3 in tobacco protoplasts (Sánchez-Navarro and Bol 2001), it was concluded that the N-terminal 11 or C-terminal 45 amino acids of the MP are dispensable for tubule formation. Moreover, a strict correlation between tubule formation in protoplasts and cell-to-cell movement in the plants of MP deletion mutants was observed (Sánchez-Navarro and Bol 2001). Transient expression of MP–GFP in epidermal cells of onion bulb scales showed that the fusion protein colocalized with the endoplasmic reticulum (ER) of transfected cells. Moreover, the protein accumulated in punctate structures in the cell wall, which probably represent plasmodesmata, and the protein was able to move into adjacent cells (Huang and Zhang 1999).

In the present study, functional domains of AMV MP were characterized further by alanine-scanning mutagenesis. Mutant MP–GFP fusion proteins were expressed transiently in tobacco protoplasts, tobacco suspension cells, and epidermal cells of tobacco leaves to investigate domains involved in tubule formation and the association of MP with the ER and

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cell wall. In addition, the mutations were introduced in the MP gene of AMV RNA 3 and the RNA was used to infect transgenic tobacco plants transformed with the AMV P_1 and P_2 genes (P_{12} plants). Some mutants defective in tubule formation were able to associate with the ER, whereas others were not. Unexpectedly, two mutants defective in ER association and tubule formation showed a low level of cell-to-cell movement in plants. Mutants that were functional in ER association and tubule formation were able to move cell to cell in plants with various efficiencies, but some of these mutants were defective in systemic movement.

RESULTS

Expression of MP mutants in tobacco protoplasts.

With the exception of M70, mutations (Table 1) were introduced in the MP sequence of the MP–GFP fusion protein encoded by plasmid pE6113MP-GFP (Zheng et al. 1997). The mutations were distributed over the MP gene, as shown schematically in Figure 1. Each mutation involved the replacement of one, two, or three amino acids, mostly by alanine residues. In some cases, amino acids were replaced by residues other than alanine to permit the introduction of a novel restriction site at each mutation.

After transfection of tobacco BY-2 protoplasts with the mutant plasmids and the wild-type plasmid as a control, the protoplasts were examined 16, 24, and 48 h posttransfection (hpt) by fluorescence microscopy. The wild-type plasmid induces the massive formation of tubular structures that protrude from the surface of the protoplasts (Zheng et al. 1997). Wild-type levels of tubule formation were obtained with mutants M4, M31, M103, M166, M179, M229, and M293 (Table 1). Results obtained with mutants M4 and M103 are shown in the top panels of Figure 2. Similar to the tubules generated with wild-type MP–GFP, the protrusions on the protoplasts expressing these mutant proteins were approximately 25 to 50 μm at 16 and 24 hpt. At 48 hpt, the protrusions could hardly be detected. Presumably, they were detached from the protoplasts or degraded (Zheng et al. 1997).

The other six mutants did not induce formation of detectable tubular structures at any time point. Fluorescence induced by mutants M53, M95, M127, and M138 accumulated mainly in the cytoplasm (Fig. 2, middle panels, and Table 1). The distribution of this fluorescence was similar to that of free GFP expressed in protoplasts from plasmid pE6113-GFP (Zheng et al. 1997). Accumulation of MP–GFP fusion proteins expressed by mutants M25 and M123, however, was confined mostly to a filamentous network localized in the cytoplasm (Fig. 2, bottom panels, and Table 1).

Expression of MP mutants in tobacco suspension cells.

Expression of the wild-type MP–GFP fusion protein in tobacco BY-2 suspension cells from plasmid pE6113MP-GFP resulted in the accumulation of the protein in filamentous structures and patch-like structures that co-localized with the ER (Huang and Zhang 1999), a distribution similar to the wild-type was observed with mutants M4, M31, M103, M166, M179, M229, and M293. A result with mutant M4 is shown in Figure 3A (full results in Table 1). Similar to their localization in protoplasts, the fluorescence of mutants M53, M95, M127, and M138 induced in suspension cells was confined mainly to the cytoplasm. The results with mutants M127, M53, and M138 are shown in Figure 3B, C, and D, respectively. The fusion proteins of mutants M25 and M123 are associated with filamentous structures, as observed with the wild-type protein (Table 1). These structures probably co-localize with the ER (Huang and Zhang 1999). The patch-like structures seen with the wild-type fusion protein, however, were not observed with mutants M25 or M123 (data not shown).

Expression of MP mutants in the epidermis cells of tobacco leaves.

In the study by Huang and Zhang (1999), localization of the wild-type MP–GFP in the epidermis cells of onion bulb scales is facilitated by the large size and absence of chloroplasts in this cell type. The use of an hexyl ester of rhodamine B as an ER-specific stain permitted the conclusion that MP–GFP was associated with filamentous structures that represent the ER. In addi-

Table 1. Genotype and phenotype of *Alfalfa mosaic virus* movement protein (MP) mutants

Mutant	Amino acids mutated	Amino acid substitutions	Accumulation in tobacco protoplasts	Accumulation in tobacco suspension cells[a]	Accumulation in tobacco epidermis cells[b]	Cell-to-cell movement in tobacco plants[c]	Systemic movement in tobacco plants
Wild type	None	None	Tubules	FIL/ER	FIL/ER, CW, AC	+++	+++
M4	4–6	TKT→AAA	Wild type	Wild type	Wild type	+++	+++
M25	25–26	EE→AA	Filaments	Filaments	FIL/ER, CW	–	–
M31	31–32	DE→AA	Wild type	Wild type	Wild type	+++	+++
M53	53–55	LVL→RYP	Cytoplasm	Cytoplasm	Cytoplasm	–	–
M70	70–72	EKK→AAA	Not determined	Not determined	Not determined	+	–
M95	95	L→A	Cytoplasm	Cytoplasm	Cytoplasm	+	–
M103	103	K→L	Wild type	Wild type	Wild type	+++	++
M123	123–125	DVD→AVG	Filaments	Filaments	FIL/ER, CW	Not determined	Not determined
M127	127–128	DH→AA	Cytoplasm	Cytoplasm	Cytoplasm	+	–
M138	138–139	GR→AA	Cytoplasm	Cytoplasm	Cytoplasm	–	–
M166	166–168	NAN→RED	Wild type	Wild type	Wild type	+++	+++
M179	179–181	EDE→AAA	Wild type	Wild type	Wild type	Not determined	Not determined
M229	229–231	KND→AAA	Wild type	Wild type	Wild type	+	–
M293	293–294	KD→AA	Wild type	Wild type	Wild type	++	–

[a] FIL/ER, filamentous structures that co-localize with the endoplasmic reticulum in tobacco suspension cells and epidermis cells of onion bulb scales.
[b] CW, punctate spots in the cell wall; AC, movement of MP fusion protein to adjacent cells.
[c] + and – represent levels of RNA accumulation (Fig. 6). +++, wild-type accumulation; ++ and +, reduced accumulation; –, no detectable accumulation.

tion, MP-GFP accumulated in punctate structures in the cell wall of transfected onion cells and in adjacent cells (Huang and Zhang 1999). In transfected tobacco epidermal leaf cells, MP–GFP also accumulated in punctate spots in the cell wall and in a network-like pattern in the cell cortex, but technical constraints prevented further characterization of this filamentous structure (Huang and Zang 1999). In the present work, transient expression of mutant MP–GFP proteins was studied in the epidermis cells of onion bulb scales and tobacco leaves. For each mutant, a similar localization was found in both types of cells, and only the results with the tobacco cells are shown.

The transient expression of mutants M4, M31, M103, M166, M179, M229, and M293 in epidermis cells resulted in a localization of the MP–GFP fusion proteins that was indistinguishable from the wild type. Examples of the expression of M4, M103, M166, and M293 in tobacco cells are shown in Figure 4. The mutants accumulated in small cell clusters at 16 to 24 hpt. Like the wild-type MP–GFP, these mutants were apparently able to move from transfected cells to adjacent cells. Mutants M25 and M123 accumulated in intracellular filamentous structures and punctate spots in the cell wall of transfected cells, but did not move to adjacent cells. Two different sections of a tobacco cell transfected with M25 and one example of a tobacco cell transfected with M123 are shown in Figure 5. The assumption that the punctate fluorescent spots in the cell wall co-localize with plasmodesmata is supported by the observation that these spots are absent in guard cells transfected with wild-type MP–GFP (Fig. 5D). Finally, mutants M53, M95, M127, and M138 accumulate in the cytoplasm of epidermis cells. No association with filamentous structures or the cell wall and no spread to adjacent cells were observed with these mutants. Examples of the accumulation of M127 and M138 in tobacco epidermis cells are shown in Figure 4 (all results in Table 1).

Previously, it was shown that MP–GFP expressed in insect cells co-localized with the ER and fully segregated with the 30,000 × *g* pellet fraction of an insect cell homogenate (Huang and Zhang 1999). To analyze the subcellular distribution of mutant M53, this mutant was expressed in insect cells and a homogenate of the cells was fractionated into a 30,000 *g* pellet (P) and supernatant (S). In contrast to the wild-type MP–GFP, 40% of the mutant protein was present in the supernatant fraction (Fig. 6). This supports the notion that the mutation interfered with the association of the MP–GFP fusion protein with the ER.

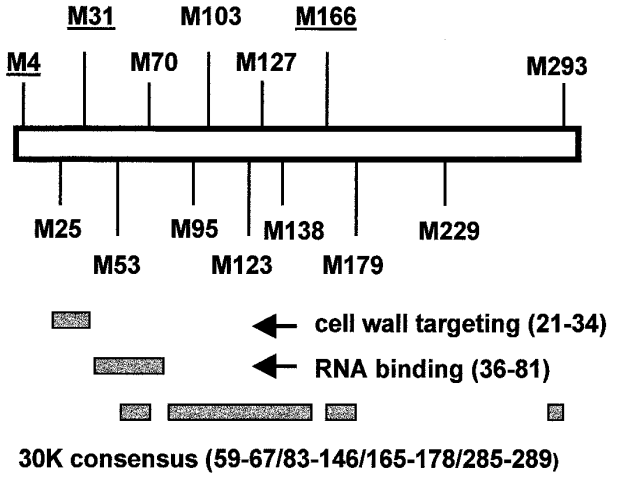

Fig. 1. Schematic representation of the location of mutations in *Alfalfa mosaic virus* movement protein. Domains (identified previously): cell wall targeting sequence (amino acids 21–34) (Berna 1995), RNA binding sequence (amino acids 36–81) (Schoumacher et al. 1994), and the 30K superfamily consensus sequence (amino acids 59–67, 83–146, 165–178, and 285–289) (Melcher 2000). Mutants that showed a wild-type phenotype in all experiments are underlined.

Expression of MP mutants in P_{12} tobacco plants.

The MP gene in AMV RNA 3 was replaced by the mutant MP genes (Table 1), with the exception of M123 and M179,

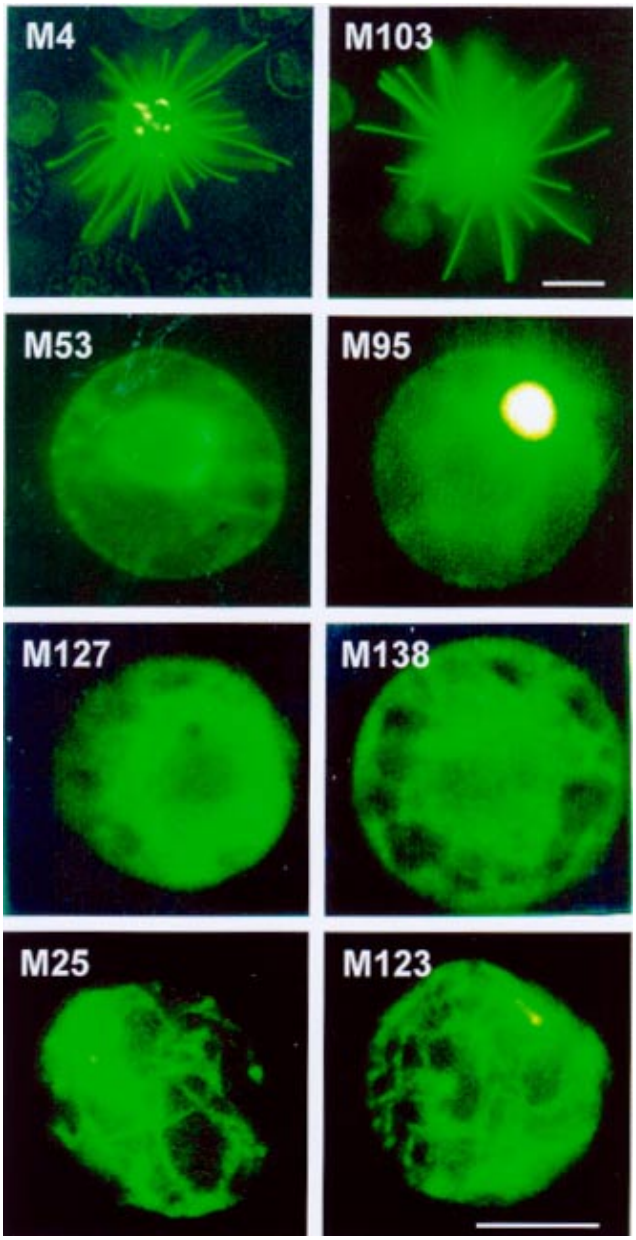

Fig. 2. Transient expression of mutant movement protein–green fluorescent protein fusions in tobacco protoplasts. Protoplasts were analyzed by inverted epifluorescence microscopy at 16 (M4 and M103), 24 (M53, M95, M127, and M138), or 48 (M25 and M123) h after transfection. Bar = 20 μm.

and mutant RNA 3 transcripts were used to infect transgenic P_{12} tobacco plants that express the AMV replicase proteins P_1 and P_2 (Taschner et al. 1991). Accumulation of viral RNA in inoculated leaves and systemically infected leaves was analyzed to monitor cell-to-cell movement and systemic movement of the mutants, respectively (Fig. 7 and Table 1). Three mutants, M4, M31, and M166, accumulated at wild-type levels in inoculated and systemically infected leaves (Fig. 7, lanes 2, 7, and 9). One mutant, M103, accumulated at wild-type levels in inoculated leaves, but accumulation of this mutant in systemic leaves was slightly reduced (Fig. 7, lane 5). The accumulation of mutant M293 in inoculated leaves was only slightly less than the wild type, but systemic accumulation of this mutant was completely blocked (Fig. 7, lane 11). Mutants M70, M95, M127, and M229 accumulated at relatively low levels in inoculated leaves, and the systemic movement of these mutants was not detectable (Fig. 7, lanes 3, 4, 8, and 10). Finally, no accumulation of mutants M25, M53, and M138 in inoculated or systemic leaves was detectable (Fig. 7, lanes 1, 6, and 12).

To rule out the possibility that the accumulation of mutants M4, M31, M70, M95, M103, M127, M166, M229, or M293 was the result of loss of the mutation, progeny RNA of these mutants was isolated from infected leaves and DNA copies of RNA 3 were amplified by reverse-transcription-polymerase chain reaction (RT-PCR). All cDNAs contained the restriction sites corresponding to the mutations (data not shown). Moreover, it was checked that all mutants accumulated at wild-type levels after infection of P_{12} protoplasts (data not shown). Thus, the mutant MPs did not inhibit RNA 3 replication.

To confirm that the accumulation of viral RNA in inoculated leaves reflected cell-to-cell movement of the virus, the MP genes of mutants M25, M31, M53, M95, M103, M123, M138, and M293 were used to replace the MP gene in the RNA 3 vector GFP/MP/CP. This vector expresses the MP gene from a subgenomic promoter, whereas the 5′ proximal GFP gene is translated from the vector RNA (Sánchez-Navarro et al. 2001). P_{12} tobacco plants were inoculated with mutant vector RNAs, and the spread of the green fluorescent signal was used to monitor cell-to-cell movement of the mutants in the inoculated leaves for 12 days after infection. Mutants M25, M53, and M138 did not reveal the spread of GFP when their MP gene was expressed from the GFP/MP/CP construct. This confirms that the absence of detectable RNA accumulation by these mutants (Fig. 7) reflect a defect in cell-to-cell movement. Also, mutant M123, which was not included in the Northern analysis (Fig. 7), did not induce the spread of GFP fluorescence. The MP genes of mutants M31, M95, M103, and M293, however, promoted cell-to-cell movement of the corresponding GFP/MP/CP constructs. Examples of green fluorescent leaf tissue infected with mutants M31 and M103 are shown in Figure 8. The rate of cell-to-cell spread of these four mutants was not quantified.

Localization of MP–GFP in tobacco protoplasts treated with cytoskeleton inhibitors.

To determine whether components of the cytoskeleton are involved in the formation of tubular protrusions or an intracellular filamentous network, transfected protoplasts were exposed to cytoskeleton inhibitors. Transfection was performed with plasmid p6113MPGFP to express a wild-type MP–GFP

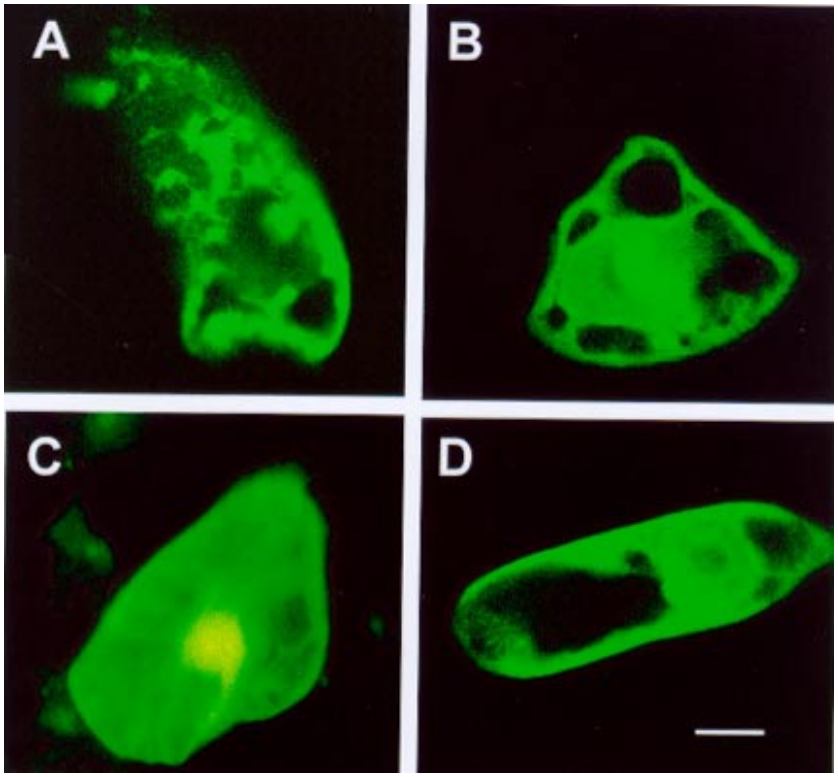

Fig. 3. Distribution of mutant movement protein–green fluorescent protein expressed in tobacco suspension cells that were transfected with mutants **A,** M4, **B,** M127, **C,** M53, or **D,** M138. The cells were analyzed 48 h after transfection by inverted epifluorescence microscopy. Bar = 20 μm.

protein. At 3 h before or immediately after transfection, the inhibitors were added and remained present in the medium during incubation of the transfected protoplasts for 16 h. Experiments with the 3-h preincubation time were performed to prevent the possibility that components of the cytoskeleton would become insensitive to the inhibitor after a possible interaction with the viral MP. When cytochalasin D (Cyt D), an inhibitor of actin polymerization, was added in concentrations of 1 to 100 μm before or after the transfection of protoplasts, no effect on tubule formation on the protoplasts surface was observed (Table 2 lists the number of fluorescent protoplasts with and without tubules at each Cyt D concentration). Tubule formation on protoplasts preincubated with 100 μm of Cyt D is shown in Figure 9A. As a control, tobacco protoplasts were transfected with plasmid p005TA to express a GFP–mouse talin fusion protein that specifically binds actin polymers and

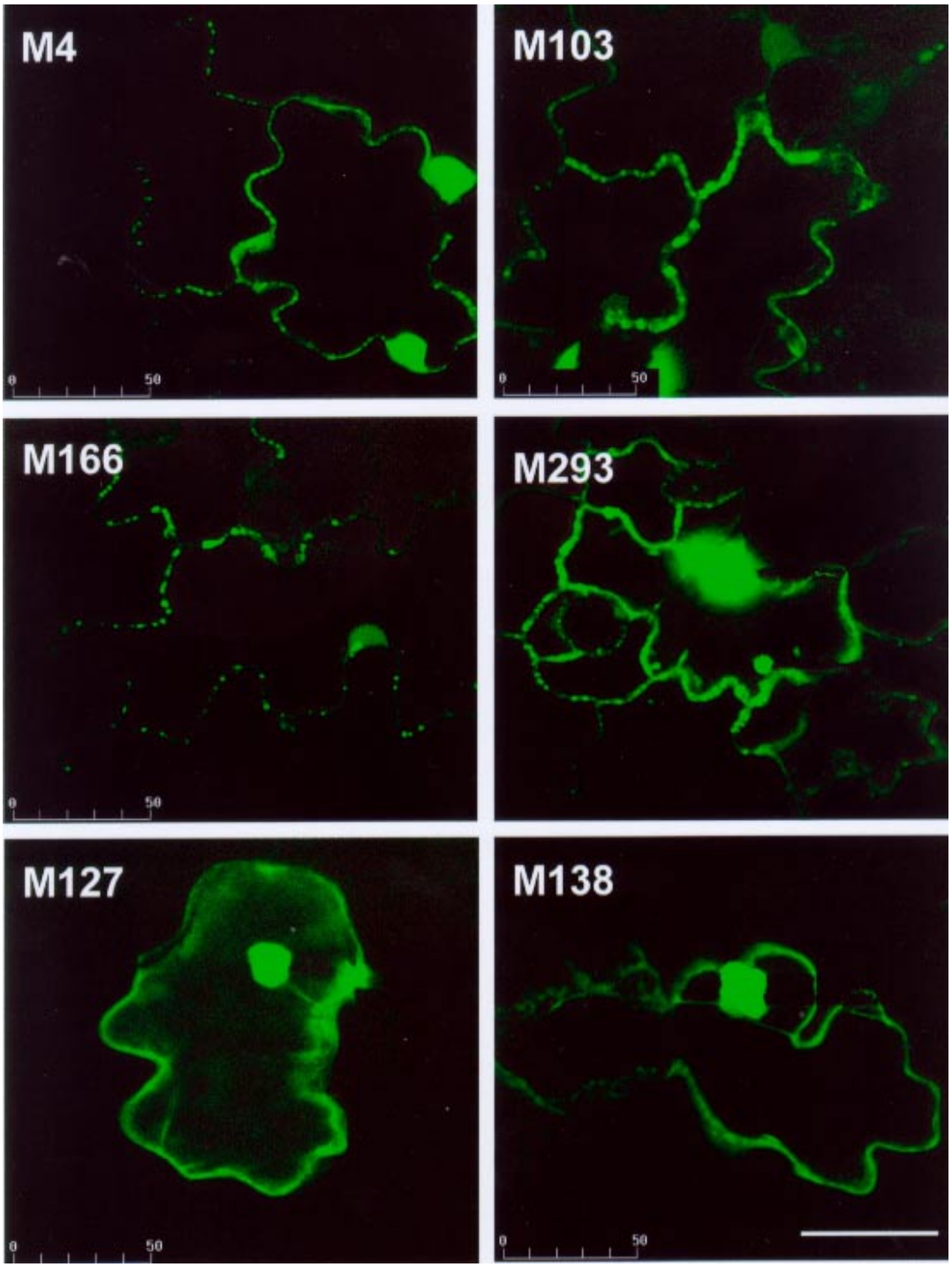

Fig. 4. Transient expression of mutant movement protein–green fluorescent proteins in the epidermis cells of tobacco leaf tissue. Images were taken by confocal laser scanning microscopy 24 h after transfection with the indicated mutants. Bar = 50 μm.

monomers (Kost et al. 1998). Fluorescent labeling of actin polymers in untreated protoplasts is shown in Figure 10A. The addition of 10 μM Cyt D completely destroyed the GFP-tagged actin filaments in the transfected protoplasts (Fig. 10B).

Latrunculin B is a stronger inhibitor of actin polymerization. The addition of this inhibitor (2.5 μM) 3 h before transfection of protoplasts with p6113MPGFP did not interfere with the formation of tubular structures on the protoplast surface (Fig. 9B). Similarly, the addition of the microtubule polymerization inhibitors colchicine at 10 μM (Fig. 9C) or oryzalin at 100 μM (Fig. 9D) 3 h before transfection of the protoplasts did not interfere with the assembly of MP–GFP into tubular structures. Identical results were obtained when the inhibitors were added after transfection of the protoplasts (data not shown).

In protoplasts, the MP–GFP of mutants M25 and M123 did not show the cytoplasmic localization observed with mutants such as M53 but induced formation of fluorescent filamentous structures. Moreover, these two mutants were defective in tubule formation (Fig. 2). In tobacco suspension cells, wild-type MP–GFP does not form tubular structures but associates with filamentous structures that were identified as the ER (Huang and Zhang 1999). Possibly, the fluorescent filamentous structures in protoplasts transfected with M25 or M123 also correspond to the ER. To determine whether these structures were sensitive to cytoskeleton inhibitors, protoplasts transfected with mutant M123 were treated with 100 μM oryzalin (Fig. 11B) or 100 μM Cyt D (Fig. 11C). The untreated control of M123-transfected protoplasts is shown in Figure 11A. The inhibitors had no effect on the formation of the fluorescent filamentous network, although some distortion of the protoplasts was observed. This may be the result of a disruption of cytoskeletal structures by the drugs. Similar results were obtained when M25-transfected protoplasts were treated with the two inhibitors (data not shown).

Previously, the hexyl ester of rhodamine B was used to stain the ER in tobacco suspension cells and the epidermis cells of onion bulb scales (Huang and Zhang 1999). Figure 12 shows the green fluorescence of MP–GFP and red staining of the ER of two tobacco protoplasts transfected with plasmid p6113MPGFP. The overlay of the pictures in the right panels of Figure 12 shows that the tubules consisting of the MP–GFP fusion protein are stained with ER-specific dye. This experiment indicates that tubular structures may contain material derived from ER membranes as well as AMV MP, and the preparations contained tubular structures that had been detached from the protoplasts. In addition, these isolated green fluorescent tubules were stained with the ER-specific dye (result not shown), suggesting that the tubules contain ER-derived material.

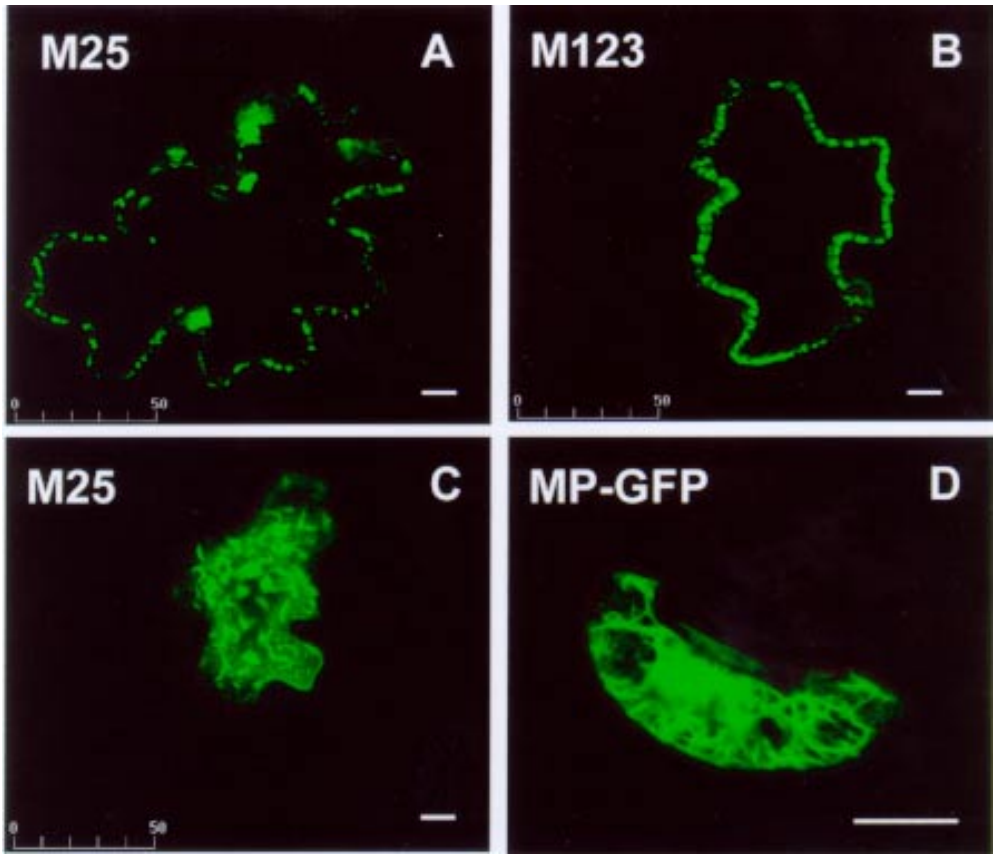

Fig. 5. Transient expression of movement protein–green fluorescent proteins (MP–GFP) in the epidermis cells and guard cells of tobacco leaves. Images were taken by confocal laser scanning microscopy 48 h after transfection. Localization of MP–GFP of **A,** mutant M25 in the cell wall and **C,** filamentous network of the cell cortex of a single transfected epidermis cell. Accumulation of **B,** mutant M123 in an epidermis cell and **D,** wild-type MP–GFP in a guard cell. Note that none of the fusion proteins moved to adjacent cells. Bar = 50 μm.

DISCUSSION

The aim of this study was to characterize domains involved in various functions of AMV MP. Sequence elements that have been recognized in the 300-amino-acid MP are indicated in Figure 1. These include a domain implicated in the targeting of the MP to the cell wall (amino acids 21 to 34) (Berna 1995; Erny et al. 1992), a domain required for nonspecific RNA binding activity (amino acids 36 to 81) (Schoumacher et al. 1994), and the consensus sequence of the 30K superfamily domain that was identified by comparison of the MPs of a number of plant viruses (amino acids 59 to 67, 83 to 146, 165 to 178, and 285 to 289) (Melcher 2000). Previously, we studied the in situ localization of the wild-type MP in epidermis cells of onion bulb scales (Huang and Zhang 1999). Although these cells offer several advantages in studies on functional domains in MPs (Knebel et al. 1990; Quader and Schnepf 1986; Quader et al. 1989), it is not known whether the onion tissue supports AMV cell-to-cell movement. Therefore, we used tobacco protoplasts, suspension cells, epidermis tissue, and plants in the present study.

The MPs of TMV and AMV have been shown to associate with the ER (Heinlein et al. 1998; Huang and Zhang 1999; Reichel and Beachy 1998). The physical continuity of the ER membrane between adjacent cells suggests a potential pathway for the movement of lipophilic molecules and membrane-associated proteins (Grabski et al. 1993). Complexes of viral ribonucleoprotein could move along the ER to plasmodesmata where interactions of MP with plasmodesmatal components could alter the size-exclusion limit of these organelles. Our studies with inhibitors of actin and microtubule polymerization indicate that the cytoskeleton is not involved in transport of AMV MP to the cell wall. Four mutant MP–GFP fusion proteins, M53, M95, M127, and M138, appeared defective in association with the ER because these proteins accumulated in the cytoplasm of protoplasts, suspension cells, and epidermis cells. The mutations of mutants M95, M127, and M138 are located in the 30K superfamily domain. Additionally, mutations in the 30K superfamily domain of *Cauliflower mosaic virus* MP and TMV or CPMV MP–GFP fusions affect intracellular targeting and resulted in cytoplasmic accumulation of the proteins (Bertens et al. 2000; Kahn et al. 1998; Thomas and Maule 1995). Although this domain may have a role in targeting the MP to the ER, the results with mutant M53 indicate that other sequences of AMV MP are involved as well. The defect in intracellular trafficking of mutants M53, M95, M127, and M138 may explain the inability of these mutants to assemble into tubular structures on the surface of protoplasts. Mutants M25 and M123 also were defective in the formation of tubular structures, but in protoplasts, these mutants accumulated in a characteristic filamentous network structure. Similar fluorescent filamentous structures are observed in suspension cells and epidermis cells transfected with wild-type MP–GFP and co-localize with the ER (Huang and Zhang 1999).

As discussed below, results with epidermis cells indicate that mutants M25 and M123 are not defective in intracellular trafficking. The MP-GFP of these mutants is probably defective in tubule formation at the protoplast surface. In wild-type MP–GFP-transfected protoplasts, fluorescent filamentous structures are clearly less detectable, probably because most of the fusion protein is recruited for tubule formation. The results indicate that these wild-type tubules contain material derived from the ER (Fig. 12). The hexyl ester of rhodamine B, however, also has been reported to stain the plasmalemma (Terasaki and Reese 1992). Thus, the presence of plasmalemma-derived material in the protrusions cannot be ruled out.

Within the family *Bromoviridae*, tubular structures have been observed in the cell walls of plants infected with *Cu-*

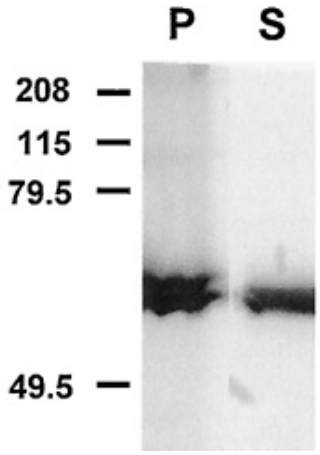

Fig. 6. Subcellular localization of movement protein–green fluorescent proteins (MP–GFP) expressed by mutant M53 in insect cells. Homogenates of the insect cells were centrifuged at 30,000 *g*, and the pellet (P) and supernatant (S) fractions were analyzed by Western blotting with an antiserum that was raised against GFP. Wild-type MP-GFP is exclusively in the pellet fraction of insect cell homogenates (Huang and Zhang 1999). Position of molecular weight markers is indicated (left).

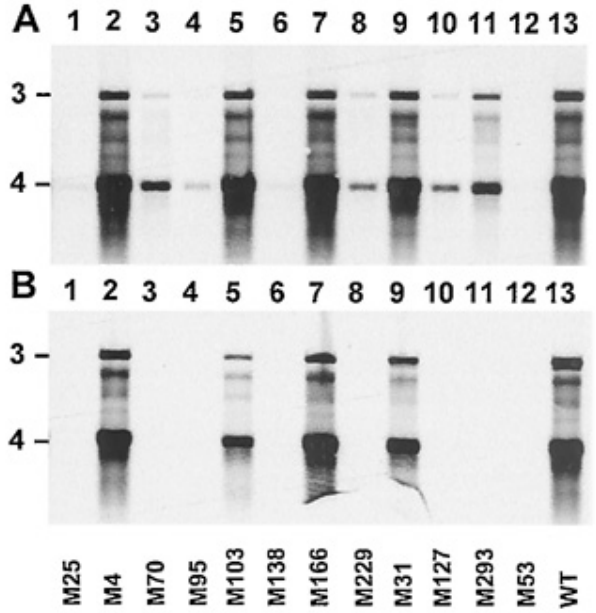

Fig. 7. Accumulation of viral RNA in tobacco plants inoculated with movement protein mutants. Transgenic P_{12} tobacco plants were inoculated with the indicated mutants or wild-type *Alfalfa mosaic virus* RNA 3. RNA was extracted from **A,** inoculated leaves or **B,** noninoculated upper leaves. RNA was analyzed by Northern blot hybridization. Position of RNAs 3 and 4 is indicated (left).

cumber mosaic virus (CMV) (Francki et al. 1985), *Tobacco streak virus* (Martelli and Russo 1985), *Olive latent virus* (Grieco et al. 1999), and AMV (Godefroy-Colburn et al. 1990). In a recent study on AMV-infected leaves of *Nicotiana benthamiana*, MP and CP could be detected in plasmodesmata in a layer of three to four cells at the front of the infection (Van der Wel et al. 1998). The desmotubule of these plasmodesmata appeared to be removed and the average diameter was increased from 20 nm in uninfected or fully infected cells to 36 nm in cells at the front of the infection. No tubular structures were observed in the modified plasmodesmata (Van der Wel et al. 1998), however, which may be the result of transient accumulation of the MP in plant tissue. Additionally, no tubular structures were observed in MP–GFP-transfected tobacco suspension cells or onion epidermis cells (Huang and Zhang 1999).

In the epidermis cells of onion bulb scales, MP–GFP was associated with the ER and punctate spots in the cell wall. These punctate spots are believed to correspond to plasmodesmata because they are absent in cells that have no connection with neighboring cells such as tobacco suspension cells or guard cells, which are believed to be symplastically isolated (Lucas and Gilbertson 1994). The MP–GFP of mutants M53, M95, M127, and M138 accumulated in the cytoplasm of tobacco suspension cells and epidermis cells of tobacco leaves. No association of MP–GFP with the ER or punctate spots in the walls of epidermis cells was observed with these mutants. The MP–GFPs of mutants M25 and M123 did associate with ER filaments and punctate spots in epidermal cell walls but were unable to move to adjacent cells. Apparently, M25 and M123 are not defective in intracellular trafficking and their defect in cell-to-cell movement may correlate with the defect in tubule formation observed in protoplasts.

Currently, the role of tubular structures in cell-to-cell movement of viruses from the family *Bromoviridae* is not clear. A CMV MP mutant that could spread systemically in tobacco has been reported, although the encoded MP was un-

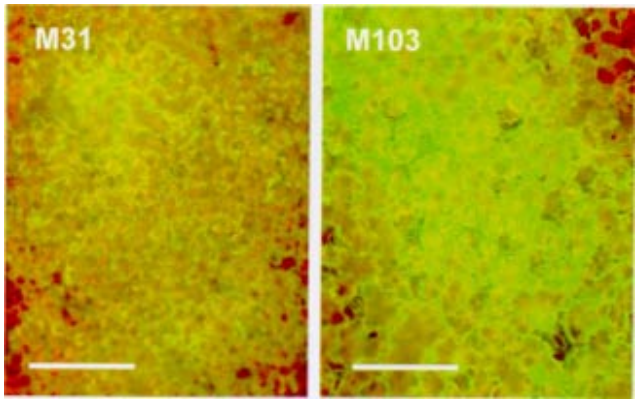

Fig. 8. Examples of fluorescent leaf tissue from P_{12} plants inoculated with the RNA 3 vector green fluorescent protein/movement protein/coat protein (GFP/MP/CP) carrying mutant MP genes M31 (left) or M103 (right). At 5 days postinoculation, green fluorescence was monitored with a confocal laser scanning microscope. Bar = 500 μm.

Table 2. Effect of cytochalasin D on tubule formation by the movement protein–green fluorescent protein (MP-GFP) fusion protein in tobacco protoplasts

	Number of fluorescent protoplasts[a]	
Cytochalasin D concentration (μM)	**Without tubular protrusions**	**With tubular protrusions**
0	32	43
1	35	41
10	29	40
50	33	45
100	32	40

[a] Tobacco protoplasts were transfected with plasmid p6113MPGFP expressing the wild-type MP-GFP fusion protein. After transfection, cytochalasin D was added and the protoplasts were incubated for 16 h. Approximately 70–80 randomly selected fluorescent protoplasts were analyzed for tubule formation by fluorescence microscopy.

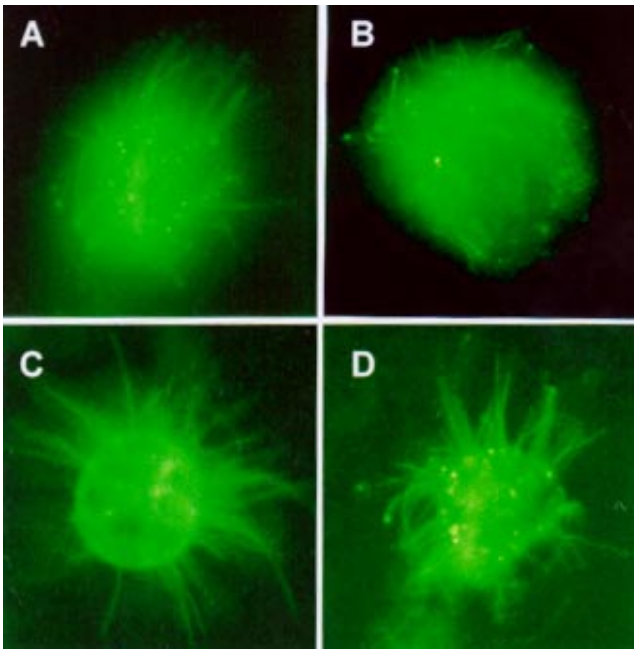

Fig. 9. Analysis of the role of the cytoskeleton in the in situ accumulation of wild-type movement protein–green fluorescent proteins in transfected tobacco protoplasts. Prior to transfection, the protoplasts were incubated with **A,** 100 μM cytochalasin D, **B,** 2.5 μM latrunculin, **C,** 10 μM colchicine, or **D,** 100 μM oryzalin. After transfection with plasmid pE6113MPGFP, the protoplasts were incubated for 16 h in medium with the indicated concentrations of inhibitors. Images were taken by inverted epifluorescent microscopy. Bar = 20 μm.

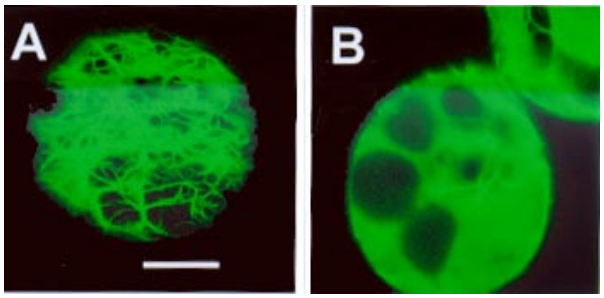

Fig. 10. Disruption of actin filaments in tobacco protoplasts by cytochalsin D. Protoplasts were transfected with plasmid p005TA to express a green fluorescent protein–mouse talin fusion protein that specifically binds actin polymers and monomers. Green fluorescence is in **A,** an untreated protoplast and **B,** a protoplast treated with 0.1 mM cytochalasin D. Images were taken by confocal laser scanning microscopy. Bar = 10 μm.

able to form tubules in protoplasts when fused to GFP. Because of this observation, the role of tubule formation in cell-to-cell movement of CMV was questioned (Canto and Palukaitis 1999). A clear correlation between tubule formation and cell-to-cell movement, however, was included in a study on AMV MP deletion mutants (Sánchez-Navarro and Bol 2001). In the present study, we observed that mutants M95 and M127 are defective in tubule formation but are able to move cell to cell in P_{12} plants, although at a strongly reduced level. We cannot rule out the possibility that a defect in tubule formation is enhanced by the GFP moiety of the mutant MP-GFP proteins expressed in protoplasts. Possibly, the nonfused MP of these mutants is capable of a low level of tubule formation in plants. The other mutants that were defective in tubule formation (M25, M53, M123, and M138) did not move cell to cell in plants.

Mutants M4, M31, and M166 showed a wild-type behavior in all functions tested in our study, whereas mutant M103 showed only a minor reduction in systemic movement in plants. Apparently, the sequences affected in these mutants are less critical for MP functioning. MP–GFP fusion proteins of mutants M229 and M293 were fully functional in association with ER, targeting to the cell wall, tubule formation, and the spread from transfected epidermis cells to adjacent cells, although cell-to-cell movement of these mutants in infected leaves was reduced and systemic spread was abolished. The mutation of M293 is located in the C-terminal 45 amino acids of MP that were dispensable for cell-to-cell movement and the systemic spread of AMV (Sánchez-Navarro and Bol 2001).

Genetic studies indicate that this C terminus contributes to a specific recognition between AMV MP and its cognate CP (Sánchez-Navarro and Bol 2001). The M293 mutation possibly interferes with a specific MP–CP interaction. Because the phenotypes of M229 and M293 are similar, the M229 mutation also might affect the putative MP–CP interaction. So far, attempts to demonstrate an interaction between wild-type AMV MP and CP in the yeast two-hybrid system with viral proteins fused to the GAL4 activation domain and the DNA binding domain yielded negative results (L. Jongejan and J. F. Bol, *unpublished results*). A possible interaction between nonfused MP and CP has not yet been investigated. Accumulation of mutant M70 in plants was very similar to that of mutants M95, M127, and M229, although the in situ localization of MP–GFP fusions of this mutant has not yet been analyzed.

In summary, several of the steps in AMV movement may be affected by our mutants, including the association of MP with the ER, followed by trafficking of MP to the cell wall (M53, M95, M127, and M138); modification of plasmodesmata, possibly by the formation of tubular structures (M25 and M123); cell-to-cell movement of putative viral RNA/MP/CP complexes through modified plasmodesmata; and entry into the vascular system (M229 and M293). The results support the proposed order of events in AMV movement.

MATERIALS AND METHODS

Construction of MP mutants.

The 14 mutations in the MP gene (Table 1) were engineered in the MP–GFP sequence of plasmid pE6113MP-GFP (Zheng et al. 1997) and the MP sequence of the infectious clone pAL3 of AMV RNA 3 (Neeleman et al. 1991) by site-directed mutagenesis with the use of specific primers and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.), according to the manufacturer's instructions. Each mutation introduced a novel restriction site in the cDNA.

Mutant MP genes M25, M31, M53, M95, M103, M123, M138, and M293 from the pAL3 plasmids also were used to replace the MP gene in derivatives 12aaGFPRNA3 or 5aaGFPRNA3 of the AMV RNA3 vector GFP/MP/CP. From the 5′ to 3′ end, this vector consists of the 5′ untranslated region (UTR) of RNA 3, the GFP gene, a duplicated subgenomic promoter, the MP gene, a subgenomic promoter, the CP gene, and the 3′ UTR of RNA 3 (Sánchez-Navarro et al. 2001). pAL plasmids containing the mutant sequences M31, M103, M138, and M293 were digested with *Xho*I–*Pvu*I, and the mutated MP sequences were isolated and ligated into 12aaGFPRNA3 digested with *Xho*I–*Pvu*I, yielding 12aaGFPM31, 12aaGFPM103, 12aaGFPM138, and 12aaGFPM293, respectively. pAL plasmids containing the mutant sequences M25 and M70 were digested with *Xho*I–*Pvu*I, and the mutated MP sequences were isolated and ligated into 5aaGFPRNA3 digested with *Xho*I–*Pvu*I, yielding 5aaGFP-

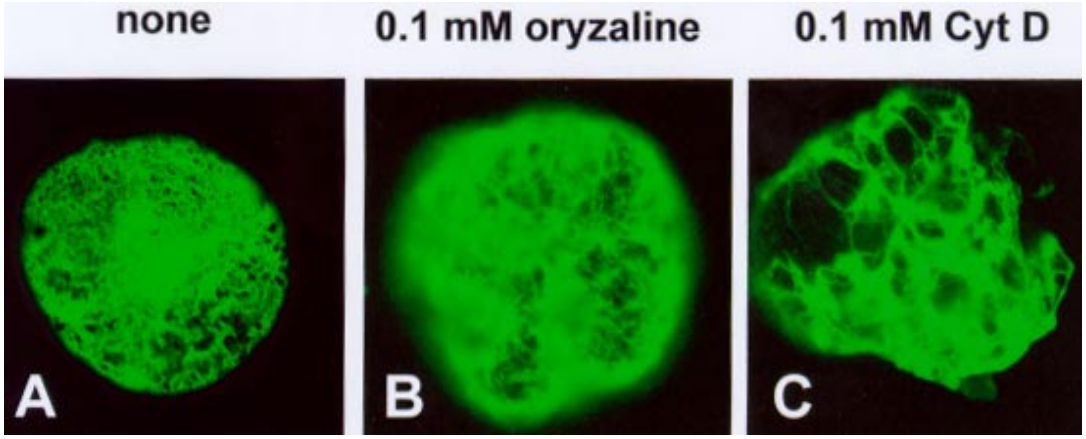

Fig. 11. Effect of oryzalin and cytochalasin D on the in situ accumulation of movement protein–green fluorescent proteins expressed transiently by mutant M123 in tobacco protoplasts. After transfection, the protoplasts were incubated for 24 h **A,** without inhibitor, **B,** with 100 μM oryzalin, or **C,** with 100 μM cytochalasin D. Images were taken by confocal laser scanning microscopy. Bar = 20 μm.

M25 and 5aaGFPM70. pAL plasmids containing the mutant sequences M53 and M123 were digested with *Xho*I–*Apa*I, and the mutated MP sequences were isolated and ligated into 5aaGFPRNA3 digested with *Xho*I–*Apa*I, yielding 5aaGFP-M53 and 5aaGFPM123. The pAL plasmid containing the mutant sequence M95 was digested with *Xho*I–*Apa*I, and the mutated MP sequence was isolated and ligated into 12aaGFPRNA3 digested with *Xho*I–*Apa*I, yielding 12aaGFP-M95.

Transfection of protoplasts, suspension cells, and epidermis cells.

Transfection of tobacco BY-2 protoplasts, tobacco BY-2 suspension cells, and the epidermis cells of onion bulb scales was performed according to the protocols described by Huang and Zhang (1999) and Zheng et al. (1997). The protoplasts were examined at 16, 24, and 48 hpt by fluorescence microscopy (Zheng et al. 1997). Transfection of tobacco epidermal cells was carried out as described by Itaya et al. (1997). A tobacco leaf was cut into four pieces, of which two were bombarded with a mutant construct and two with the wild-type construct pE6113MPGFP as a control. The bombarded leaves were cultured at room temperature in small Falcon 3001 dishes containing two layers of 3-mm paper wetted with Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands). The cells were examined by confocal laser scanning microscopy at 24 and 48 hpt.

Staining of ER and fluorescence microscopy.

The ER of onion and tobacco epidermal cells and BY-2 suspension cells was stained with the fluorescent hexyl ester of rhodamine B at a concentration of 1 mg per ml, as described by Grabski et al. (1993). Fluorescence microscopy was carried out as described by Huang and Zhang (1999).

Recombinant Baculovirus system.

Mutant M53 was expressed in a recombinant Baculovirus system. Generation of the recombinant virus vector, subsequent amplification of virus particles and extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting of the proteins was performed as described by Huang and Zhang (1999).

Inoculation and analysis of P_{12} tobacco protoplasts and plants.

Wild-type and mutant RNA3 transcripts were synthesized with T_7 RNA polymerase on plasmids that were linearized with *Sal*I (Van der Kuyl et al. 1991). Isolation and inoculation of protoplasts from P_{12} tobacco plants with wild-type and mutant transcripts were performed essentially as described previously (Loesch-Fries et al. 1985; Van Dun et al. 1988). Approximately 2.5×10^5 protoplasts per sample were inoculated by the polyethylene glycol method with the use of 30 µl of transcription mixture with inoculum RNAs. Protoplasts were incubated for 18 h at 25°C under continuous illumination.

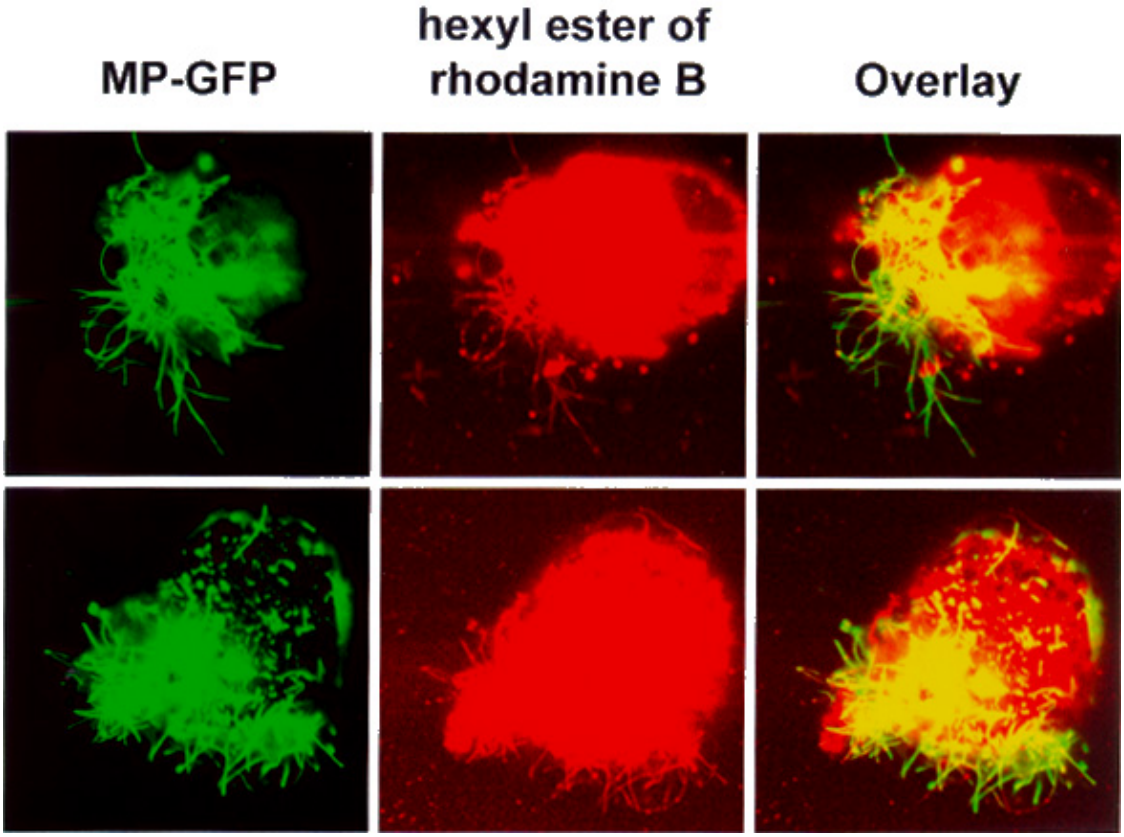

Fig. 12. Staining of tubular structures protruding from movement protein–green fluorescent proteins transfected protoplasts with the hexyl ester of rhodamine B. Left panels, two different tobacco protoplasts analyzed for green fluorescence 16 h after transfection with plasmid pE6113MPGFP. Middle panels, the same two protoplasts stained with the endoplasmatic reticulum-specific dye. Right panels, a merge of left and middle panels. Images were taken by confocal laser scanning microscopy. Bar = 20 µm.

Transgenic P_{12} tobacco plants were grown and inoculated as described previously (Neeleman et al. 1993; Taschner et al. 1991). Inoculation was performed on three leaf halves; two plants per sample were used.

Virus particles were isolated from inoculated leaves 5 days postinoculation (dpi) or from systemically infected leaves 10 dpi, as described by Van Vloten-Doting and Jaspars (1972). Total RNA was extracted from 0.5 g of infected tissue and analyzed by Northern blot hybridization, as described by Van der Kuyl et al. (1991). A radioactively labeled DNA fragment of the CP gene was used as probe. RNA was isolated from protoplast samples (2.5×10^5 protoplasts) with TRIzol reagent, according to the manufacturer's instructions (GIBCO-BRL, Rockville, MD, U.S.A.). Per protoplast sample, 0.5 ml of TRIzol reagent was used. RNA was collected from the water phase by isopropanol precipitation and resuspended in water.

Sequence analysis of progeny virus RNA.

RNA was isolated from systemically infected leaves, and a DNA copy of RNA 3 was amplified by RT-PCR with the appropriate primers. The PCR reaction contained 250 μM of each of the four deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany), 2 μl of viral RNA, and Boehringer *Taq* polymerase buffer. PCR was performed for 35 cycles at 94°C (1 min), 55°C (1 min), and 72°C (1.5 min) on a PerkinElmer DNA thermal cycler (Wellesley, MA, U.S.A.). The amplified cDNA was checked for the presence of introduced nucleotide changes by restriction enzyme analysis.

Treatment of cells with cytoskeleton inhibitors.

The actin polymerization inhibitors Cyt D and latruculin B and the microtubule polymerization inhibitors colchicine and oryzalin were used to treat protoplasts 3 h before or immediately after transfection. Before transfection, the protoplasts were preincubated with 100 mM Cyt D for 3 h, transfected with p6113MPGFP, and subsequently incubated in medium containing 100 mM Cyt D for 16 h. After transfection, MP–GFP expressing protoplasts were treated immediately with different concentrations of Cyt D (1, 5, 10, 50, or 100 mM) and the protoplasts were studied for the formation of surface protrusions. Dimethyl sulfoxide (0.1 mM), the solvent of CytD, was added to the control. After incubation for 16 h, the living protoplasts were observed by an inverted epifluorescence microscope.

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