Association of the Movement Protein of Alfalfa Mosaic Virus with the Endoplasmic Reticulum and Its Trafficking in Epidermal Cells of Onion Bulb Scales

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To study the intercellular trafficking potential of alfalfa mosaic virus movement protein (AIMV MP) and the cellular structures that may assist in this activity, the AIMV MP gene was fused with the green fluorescent protein (GFP) gene and delivered into epidermal cells of onion bulb scales and *Nicotiana tabacum* (BY-2) suspension culture cells by biolistic gene bombardment. Its subcellular distribution as well as intracellular and intercellular trafficking in epidermal cells of onion bulb scales and in insect cells were systematically studied. Cells expressing the MP-GFP showed a fluorescent filamentous network in the cell cortex and fluorescent irregular bodies scattered in the cytoplasm and present in the cell wall. By 44 h post transfection with the MP-GFP construct, 56% of fluorescent sites consisted of clustered cells, whereas the fluorescent sites from cells bombarded with the free GFP construct consisted of isolated cells. Time course experiments demonstrated that the MP-GFP was able to move into adjacent cells from a primarily transfected cell. Optical serial sectioning studies suggested that the MP-GFP could target to the plasmodesmata, and move through them into neighboring cells. Furthermore, detailed fluorescence microscopy analyses of both tobacco and onion cells showed that the MP-GFP co-localized with the endoplasmic reticulum (ER). Subcellular fractionation of insect cells and immunoblotting analysis revealed that the MP-GFP behaves as an integral membrane protein. These results indicate that the MP-GFP is associated with the ER and suggest that the ER association may be important for intracellular and intercellular movement of the AIMV MP.

One of the most important steps in the process of plant virus infection is the spread of virus from an initially infected cell to the surrounding, noninfected cells. The so-called movement protein (MP) encoded by plant viruses interacts with the plasmodesmata (Pd) and mediates virus cell-to-cell movement (for reviews see Atabekov and Taliansky 1990; Robards and Lucas 1990; Deom et al. 1992; Waigmann et al. 1994; Lucas 1995; Carrington et al. 1996; Mezitt and Lucas 1996). This

hypothesis was supported by experiments with fluorescein isothiocyanate-labeled MPs from several viruses, including red clover necrotic mosaic virus, tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), and bean dwarf mosaic virus (Fujiwara et al. 1993; Waigmann et al. 1994; Ding et al. 1995; Noueiry et al. 1994).

Alfalfa mosaic virus (AIMV) is a member of the *Bromoviridae*, a family of plant viruses with a tripartite, single-stranded, positive-sense RNA genome. Three genomic and one subgenomic RNA species are involved in the synthesis of viral structural and nonstructural proteins. The available evidence shows that the AIMV MP is encoded by RNA 3. The MP contains RNA-binding activity (Schoumacher et al. 1992a, 1992b, 1994) and can induce a limited increase of the size exclusion limit of Pd (Poirson et al. 1993). It is likely that the AIMV MP may interact with Pd and modify them to allow viral particles or viral RNA-protein complexes to transport to surrounding cells. The involvement of this protein in the spread of AIMV infection has also been documented (Stussi-Garaud et al. 1987; Dore et al. 1991; van der Kuyl et al. 1991; van der Vossen et al. 1995). However, the mechanism behind the intercellular and intracellular trafficking of the AIMV MP is still poorly understood.

Two hypothetical pathways, i.e., a TMV-like mechanism and a tubule-based mechanism, have been proposed to explain the cell-to-cell movement of plant viruses (Carrington et al. 1996). This proposal was based on the observations that the MPs of cowpea mosaic comovirus and several other viruses could induce the formation of tubular structures in both protoplasts and cultured insect cells, but the TMV MP could not form such structures (Carrington et al. 1996, and references therein). Results reported previously showed that the AIMV MP could also induce the formation of tubular structures in protoplasts, suggesting that AIMV might move from cell to cell via these tubular structures (Zheng et al. 1997). Further examination of the intracellular and intercellular distribution of the AIMV MP in plant cells by microscopy may help to reveal the natural cellular pathways of AIMV cell-to-cell movement. For this purpose, the AIMV MP was expressed as a fusion protein with the green fluorescent protein (GFP) and its subcellular distribution patterns were examined in detail by epifluorescent microscopy and confocal laser scanning microscopy (CLSM). The results reported here demonstrate that the MP-GFP co-localizes with the endoplasmic reticulum (ER) in tobacco and onion cells. However, the MP-GFP could

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not induce the formation of filamentous structures in cultured insect cells. These data suggest that the AIMV MP might mediate viral cell-to-cell movement via its association with the ER.

RESULTS

Distribution of the AIMV MP-GFP in living epidermal cells of tobacco leaves and onion bulb scales.

Analysis of the AIMV MP distribution patterns in living leaves should provide useful information on its intracellular and intercellular movement pathways. In this study, we initially analyzed the distribution patterns of the AIMV MP in tobacco (*Nicotiana tabacum* cv. Samsun) leaves transfected with pE6113MPGFP by particle bombardment. Plasmid pE6113MPGFP, which contains the MP-GFP fusion construct, was made as previously described (Zheng et al. 1997). The transfected tobacco cells were examined by CLSM at 24 and 48 h post transfection (pt). The MP-GFP was shown to be localized mainly in the cytoplasm as irregular aggregates in single cells or in small cell clusters (composed of 3 to 5 adjoined cells) at 24 h pt (e.g., Fig. 1A). Larger cell clusters (containing 7 to 15 cells) with the MP-GFP accumulated in the cell wall were seen at 48 h pt (e.g., Fig. 1B). In some cases, network-like structures were also observed in the cell cortex (Fig. 1C). In control experiments, leaves transfected with pE6113GFP, a vector expressing GFP only (Zheng et al. 1997), showed a distribution of “free” fluorescence (Fig. 1D); the fluorescence remained in single cells at 24 and 48 h pt (Fig. 1D). The networklike pattern of the MP-GFP observed in the cell cortex suggested that the MP-GFP may be associated with some subcellular membrane structures, e.g., the ER. However, detailed analysis of the distribution patterns of the MP-GFP in this cell type was hindered by certain technical constraints.

The epidermis of living onion bulb scales was then chosen in order to study the detailed distribution patterns of the MP-GFP. Subcellular structures, including the ER and cytoskeleton, of this type of cells have been well described (Quader and Schnepf 1986; Quader et al. 1989; Knebel et al. 1990). In addition, the large size and the absence of chloroplasts in this cell type facilitate examination by fluorescence microscopy. Epidermal cells of onion bulb scales were transfected with pE6113MPGFP and examined at 4-h intervals pt. At 4 h pt, the majority of the MP-GFP appeared around the nucleus with weaker fluorescence in the cytoplasm as scattered amorphous patches (Fig. 2A), morphologically resembling the ER cisternae (Quader and Schnepf 1986). At 16 h pt, more intense fluorescence was observed in individual cells with multi-shaped, fluorescent aggregates (Fig. 2B). The aggregates existed as irregular patches, single or bundled filamentous strands, and punctate spots that accumulated in the cell wall (Fig. 2B). A few of these sections also showed fluorescence in their neighboring cells with strong fluorescent spots distributed within the cell wall and very weak fluorescence in the cytoplasm (Fig. 2B). By continuous optical sectioning with CLSM, the detailed morphological characters of the MP-GFP aggregates were revealed. Although different patterns of MP-GFP distribution in each cell were observed, the MP-GFP aggregates can be classified into the following three general types. The first type of MP-GFP aggregate was typified by fluorescent patches composed of either a mass of fine filamentous elements (Fig. 2C), or clusters of fluorescent, granular particles

(Fig. 2D). These granular particles were tethered by fine filaments that rooted on some invisible structures and were seen in slight, very quick vibration (data not shown), a characteristic of membrane tubules (Tabb et al. 1998). The second type of MP-GFP aggregate was characterized by long, tubulelike elements with bulblike structures at their tips (Fig. 2E), resembling both the tubule structure termed snaking rodlike ER in soybean root cells (Grabski et al. 1993) and the tubulovesicular organelle from the squid axoplasm described by Tabb et al (1998). This wormlike shape for the MP-GFP-labeled structure can be differentiated from other cytoskeletal filaments that were generally stiff and inflexible (Tabb et al. 1998). Some of these tubulelike elements branched out and extended to the cell cortex (Fig. 2F). The third type of MP-GFP aggregate was shown as a peripheral filamentous network (Fig. 2G), which is very much like the polygonal network of the ER. This filamentous network became more prominent with increasing incubation time.

Association of the AIMV MP-GFP with the ER.

The three distribution patterns of AIMV MP-GFP described above resemble the ER network in epidermal cells of onion bulb scales (Quader and Schnepf 1986; Quader et al. 1989; Knebel et al. 1990), suggesting that the MP-GFP may be associated with the ER. To investigate this possibility, the MP-GFP-expressing onion cells were stained with the hexyl ester of rhodamine B, a short-chain carbocyanine dye known to stain specifically the ER and mitochondria (Grabski et al.

Fig. 1. Distribution of the movement protein–green fluorescent protein (MP-GFP) in epidermal cells of tobacco leaves transfected with pE6113MPGFP. **A**, MP-GFP expression in a single cell of a small cluster of tobacco epidermal cells at 24 h post transfection (pt), showing punctate irregular aggregates in the cytoplasm. **B**, MP-GFP expression in a large cluster of tobacco cells at 48 h pt, showing the accumulation of punctate irregular aggregates in the cell walls. **C**, MP-GFP expression in a tobacco epidermal cell at 48 h pt, showing networklike structures at the cell cortex. **D**, GFP expression in an epidermal cell of tobacco leaves at 48 h pt, showing typical “free” distribution. Micrographs were taken by confocal laser scanning microscopy. Bar: 20 μ m.

1993; Boevink et al. 1996). Examples of CLSM images taken from serial sections of MP-GFP-expressing cells of onion epidermis at 16 h pt are shown in Figure 3A–C. The green fluorescence represents the MP-GFP in aggregates composed of filamentous elements in the cytoplasm (Fig. 3A), and the red fluorescence represents the ER network in the same section of the cell (Fig. 3B). Merging of the two fluorescent patterns is presented in Figure 3C, showing close co-alignment of the

two patterns. The peripheral network of the MP-GFP was also co-aligned with the ER tubular network in the cell cortex (Fig. 3D–F).

Tobacco BY-2 cells were then used to test whether the MP-GFP also co-localized with the ER in a known host cell for AIMV. As shown in Figure 3G–I, co-alignment of the MP-GFP with the ER in BY-2 cells was seen, suggesting that the MP-GFP may be associated with the ER in both cell types.

Fig. 2. Distribution of the movement protein–green fluorescent protein (MP-GFP) in epidermal cells of onion bulb scales transfected with pE6113MPGFP. **A**, MP-GFP expression in an onion bulb epidermal cell at 4 h post transfection (pt), showing brighter fluorescence occurring at nuclear membrane and weaker fluorescence in cytoplasm. **B**, MP-GFP expression in onion epidermal cells at 16 h pt, showing brighter fluorescence in a central cell and weaker fluorescence in neighboring cells with punctate fluorescence regularly distributed in the cell walls. **C**, A fluorescent patch composed of filamentous elements in an onion epidermal cell at 16 h pt. **D**, A group of fluorescent granule particles in an onion epidermal cell at 16 h pt. **E**, Fluorescent, snaking, rodlike, filamentous elements in an onion epidermal cell at 16 h pt. **F**, Long, fluorescent tubules extended to and branched out at the cell cortex of an onion epidermal cell. **G**, Fluorescent filamentous network located in the cell cortex of an onion epidermal cell at 22 h pt. Photo in **B** taken with an epifluorescent microscope (Olympus Ix70, Tokyo); the rest were taken by confocal laser scanning microscopy. Bar: (**A–F**) 20 μm; (**G**) 10 μm.

Intracellular trafficking of the AIMV MP-GFP in epidermal cells of onion bulb scales.

As described above, at 16 h pt, transfected onion bulb cells showed strong fluorescent signals located both in the cyto-

plasm and in punctate distribution along the cell wall (Fig. 2B). With time, the punctate fluorescence became more obvious in the cell wall (compare Figure 2A and B). Concurrently, the cytoplasmic accumulation of the MP-GFP was reduced;

Fig. 3. Optical sectioning analysis of the co-alignment of the movement protein-green fluorescent protein (MP-GFP) with the endoplasmic reticulum (ER) in onion epidermal cells and tobacco BY-2 cells. **A**, Fluorescent patch composed of filamentous elements in lateral cross section of an onion epidermal cell transfected with pE6113MPGFP at 16 h post transfection (pt). **B**, ER membranes stained with the hexyl ester of rhodamine B in the same section shown in **A**. **C**, Overlaying of **A** and **B**, showing co-localization of the MP-GFP with the ER. **D**, Fluorescent filamentous network in a lateral cross section of an onion epidermal cell transfected with pE6113MPGFP at 44 h pt. **E**, Same section shown in **D**, showing the ER polygonal network after staining with the hexyl ester of rhodamine B. **F**, Overlaying of **D** and **E**, showing co-alignment of the MP-GFP with the ER peripheral tubular network. **G**, Fluorescent network of the MP-GFP in a BY-2 cell at 16 h pt. **H**, ER membranes stained with the hexyl ester of rhodamine B in the same section shown in **G**. **I**, Overlaying of **G** and **H**, showing co-localization of the MP-GFP with the ER. All images were collected by confocal laser scanning microscopy. Bar: (**A**–**C**, **G**–**I**) 10 μ m; (**D**–**F**) 50 μ m.

the fluorescence could be detected only at the cell cortex examined by serial, lateral cross sections collected from top to bottom through the whole cell. These observations suggest that the MP-GFP may be able to travel from the central part to the peripheral part of the cell. This possibility was supported by frequent observations, with CLSM, of the MP-GFP streaming with the cytoplasm of the transfected cells (data not shown).

Intercellular trafficking of the AIMV MP-GFP in epidermal cells of onion bulb scales.

Movement of the MP-GFP from cell to cell was analyzed by a detailed time course experiment. After particle bombardment with pE6113MPGFP, the epidermis of onion bulb scales was examined at 16, 24, and 44 h pt. Cells expressing the MP-GFP either as single cells or in cell clusters (three or

more adjacent cells showing fluorescence) were separately counted. Figure 4A shows an example of a cluster of the MP-GFP-expressing cells at 44 h pt. As shown in Table 1, an increase in the number of fluorescent clusters of MP-GFP-expressing cells was observed with increasing incubation time. By 44 h pt, approximately 56% of the fluorescent sites contained clusters of MP-GFP-expressing cells. In contrast, onion epidermal cells that were bombarded with pE6113GFP showed fluorescent signals limited to the transfected cells throughout the time course (Table 1 and Fig. 4B). Occasionally, two adjacent cells with GFP expression were observed (data not shown). Since this expression pattern remained unchanged with incubation time, it was likely that this GFP expression pattern was produced by concurrent transfection of the two adjacent cells. The fuzzy fluorescence surrounding the illuminated cell in Figure 4B may be due to an artifact of light reflection, as this phenomenon is frequently observed in non-transfected onion bulb epidermal cells. These results suggest that the majority of the fluorescent cells in the cell clusters may reflect the trafficking of the MP-GFP from a central transfected cell to the surrounding cells. Similar results were also reported for the CMV MP (Itaya et al. 1997).

The MPs of certain plant viruses were frequently shown to be localized to punctate sites in the cell wall. These punctate sites were identified as Pd (Tomenius et al. 1987; Linstead et al. 1988; Shanks et al. 1989; Wolf et al. 1989; Atkins et al. 1991; Ding et al. 1992a; Moore et al. 1992; Waigmann et al. 1994; Waigmann and Zambryski 1995; Dickinson et al. 1996; Santa Cruz et al. 1998). In this study, fluorescent MP-GFP located in punctate sites with strands in and crossing the cell wall was clearly observed in onion epidermal cells (Fig. 5A–C). Co-localization of the MP-GFP fluorescence with the punctate sites in the cell walls was visualized by staining with the hexyl ester of rhodamine B (Fig. 5D–F), indicating that the MP-GFP is also associated with Pd. This was further supported by continuous optical sectioning studies with CLSM. As shown in Figure 5G–I, the peripheral network of the MP-GFP in a central cell was connected to the peripheral network of adjacent cells via the punctate spots.

As these punctate spots may represent desmotubules, we reasoned that the MP-GFP might be able to migrate to the neighboring cells through Pd by its association with the ER. To investigate this possibility, onion cells expressing the MP-GFP were treated with 1 M Tris-HCl (pH7.0) buffer for 2 to 5 min to separate the cytoplasm from the cell wall by dehydration. The results showed that the MP-GFP was present as filamentlike structures that protruded from the plasmolyzed plasma membrane and connected adjoining cells through

Fig. 4. Distribution of the movement protein–green fluorescent protein (MP-GFP) in a cluster of onion epidermal cells transfected with (A) pE6113MPGFP and (B) pE6113GFP at 44 h post transfection (pt), showing the punctate distribution of (A) the MP-GFP in the cell walls and (B) “free” GFP in a single cell. The fuzzy fluorescence next to the GFP-expressing cell is due to artificial light reflection (see text). Photos were taken with an epifluorescent microscope. Bar: 50 µm.

Table 1. Intracellular trafficking of the alfalfa mosaic virus movement protein (AIMV MP)–green fluorescent protein (GFP) fusion protein in epidermal cells of onion bulb scales

Incubation time (h post transfection)	pE6113MPGFP		pE6113GFP	
	Single cell	Cell cluster ^a	Single cell	Cell cluster ^a
16	71	3 (4%)	123	0
24	55	22 (28%)	124	0
44	32	40 (56%)	127	0

^a A cell cluster was defined as three or more contiguous cells showing green fluorescence.

punctate sites in the cell wall (Fig. 6A). Nontransfected onion bulb cells stained with the hexyl ester of rhodamine B and treated with 1 M Tris-HCl (pH 7.0) showed very similar filamentous structures in adjoining cells (Fig. 6B). These filaments were also connected to punctate sites in the cell wall (Fig. 6B). As rhodamine B might also stain the plasmalemma (PL) (Terasaki and Reese 1992), we could not

completely rule out the possibility that these punctate spots may contain PL. However, as extensive use of this dye in our studies showed only very weak, if any, PL staining (see Figure 3), the majority of these red fluorescence spots probably reflect the staining of the ER. These results lend support to the notion that the ER association of the MP-GFP may assist its intercellular movement.

Fig. 5. Accumulation of the movement protein–green fluorescent protein (MP-GFP) at punctate sites in the cell walls of onion epidermal cells. **A**, Lateral cross section showing GFP-labeled fluorescent filamentous strands in the cell walls at 44 h post transfection (pt). **B**, Higher magnification of the boxed area shown in **A**, showing a fluorescent filamentous strand structure. **C**, Lateral cross section of the cell wall, showing the punctate distribution of MP-GFP in the cell wall of the same cell. **D**, Three contiguous MP-GFP-expressing cells, showing the MP-GFP-labeled periphery filamentous network in the cortex and MP-GFP-labeled punctate sites in the cell walls (arrowheads). **E**, The three contiguous cells shown in **D** were stained with the hexyl ester of rhodamine B, showing the peripheral endoplasmic reticulum (ER) tubule network and punctate sites in the cell wall (arrowheads). **F**, Overlaying of **D** and **E**, showing co-localization of the MP-GFP with the peripheral ER and the punctate sites in the cell walls. **G**, Lateral cross section of three adjacent cells, showing peripheral tubular network of the MP-GFP in the cell cortex and punctate distribution of the MP-GFP in the cell walls (arrowheads). **H**, Staining of the same cells shown in **G** with the hexyl ester of rhodamine B, showing the ER distribution pattern and the punctate spots in the cell walls (arrowheads). **I**, Overlaying of **A** and **B**, showing co-localization between the MP-GFP and plasmodesmata-like structures (arrowheads). All images were taken with confocal laser scanning microscopy. Bar: (**A**, **D–I**) 50 μ m; (**B** and **C**) 10 μ m.

Subcellular fractionation and immunoblot analysis of the AIMV MP-GFP expressed in insect cells.

The close association of the MP-GFP with the ER suggests that the MP-GFP is most likely a membrane-associated protein. Subcellular fractionation and immunoblotting experiments were then performed to address this possibility. As the quantity of the MP-GFP produced in the transient expression plant cells was limited, the MP-GFP and GFP were expressed in insect cells by a recombinant baculovirus system. Two recombinant baculoviruses, BacMPGFP and BacGFP, were produced by replacing the *Autographa californica* nuclear polyhedrosis virus polyhedrin gene with the MP-GFP fusion gene and the GFP gene, respectively. Protein extracts were prepared from the insect cells infected with BacMPGFP and were analyzed by immunoprobe blots with anti-GFP antibody. A control experiment was carried out in parallel with insect cells infected with BacGFP. As shown in Figure 7A, a single 59-kDa polypeptide, representing the MP-GFP, was specifically

detected in the P30 fraction (Fig. 7A, lane 2); only trace amounts of the same protein were detected in the S30 fraction (Fig. 7A, lane 1). These results showed that most of the AIMV MP was not present as a soluble cytoplasmic protein. In contrast, in the extract made from BacGFP-infected Sf9 cells, a 27-kDa protein representing the native GFP was detected in the S30 fraction (Fig. 7A, lane 3), but not in the P30 fraction (Fig. 7A, lane 4), indicating that the native GFP was present as a soluble cytoplasmic protein in insect cells.

The P30 fraction containing the MP-GFP was then exposed to different biochemical treatments. The results showed that MP-GFP could not be extracted from the membrane preparation upon treatments with 1 M NaCl (Fig. 7B, lane 1), 1 M KCl (Fig. 7B, lane 2), 0.1 M NaHCO₃ (pH 11) (Fig. 7B, lane 3), or 0.15 M Na₂CO₃ (pH 11.5) (Fig. 7B, lane 4), respectively. However, some, but not all, of the MP-GFP was released from the membranes by extraction with either 1% NP-40 (Fig. 7B, lane 5) or 1% Triton X-100 (Fig. 7B, lane 6), suggesting that the MP-GFP was integrally associated with the membrane structures.

Intracellular distribution of the AIMV MP-GFP in insect cells.

The intracellular localization of the MP-GFP in insect cell was examined daily after infection of Sf9 cells with recombinant baculovirus BacMPGFP. The infected cells showed the formation of MP-GFP-labeled amorphous patches in the cyto-

Fig. 6. Treatment of the (A) movement protein–green fluorescent protein (MP-GFP) expressing cells and (B) nontransfected cells with 1 M Tris-HCl. The MP-GFP-labeled strands connecting the cytoplasm of contiguous cells through punctate sites in the cell walls are shown in A. Photos were taken with an epifluorescent microscope. Bar: 60 μ m.

Fig. 7. Association of the movement protein–green fluorescent protein (MP-GFP) with insect cell membranes. **A**, Preparation of the (lanes 1 and 3) S30 and (lanes 2 and 4) P30 fractions from Sf9 insect cells infected with either (lanes 1 and 2) BacMPGFP or (lanes 3 and 4) BacGFP. Polypeptides were separated on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel and visualized by Western blotting (immunoblotting) and immunoprobe with anti-GFP antiserum. Numbers indicate molecular masses in kilodaltons. **B**, Chemical treatments of P30 fractions. Aliquots of the membrane preparation were exposed to (lane 1) 1 M NaCl, (lane 2) 0.1 M NaHCO₃, (lane 3) 1 M KCl, (lane 4) 0.15 M Na₂CO₃, (lane 5) 1% NP-40, and (lane 6) 1% Triton X-100, respectively. Polypeptides in supernatants (S) and pellets (P) were separated on an SDS-12% polyacrylamide gel, and the MP-GFP was visualized by Western blotting and immunoprobe with anti-GFP antiserum.

plasm 2 days postinfection (pi); insect cells infected with BacGFP showed the typical distribution pattern of “free” GFP (data not shown). At 4 days pi, most of the infected cells exhibited an irregular patchwork connected with fine filaments. These patchworks were visualized to accumulate in the cell cortex by serial optical sectioning through whole cells with CLSM. One example showing that the MP-GFP accumulated in the cell cortex is shown in Figure 8A. Immunostaining with anti-BiP (an ER lumen protein) antibody and rhodamine labeled anti-IgG showed that the MP-GFP expressed in Sf9 cells co-localized with the ER (Fig. 8A–C).

DISCUSSION

Epidermal cells of onion bulb scales have been used widely as representative of other cell types of higher plants to study the ER and cytoplasmic streaming (Quader and Schnepf 1986; Quader et al. 1989; Knebel et al. 1990). Although it is not known whether the onion epidermal cell is susceptible to infection by AIMV, the similar distribution patterns of the MP-GFP observed in tobacco epidermal cells, BY-2 cells, insect cells, and onion epidermal cells suggest that the MP-GFP may be associated with a very conserved subcellular structure. The co-localization of the MP-GFP with the ER in both BY-2 and onion epidermal cells presented in this report may represent a genuine pathway used by the AIMV MP in mediating cell-to-cell movement of AIMV.

Although the MP gene does not contain a specific, known ER targeting signal, the co-localization of the MP-GFP with the ER in both plant and insect cells suggests that the MP-GFP is closely associated with the ER. The integral membrane protein property of the MP-GFP further supports this notion. As free GFP is a soluble protein not specifically associated with cellular membrane structures, the ER-anchoring signal sequence in the MP-GFP must be contributed by the AIMV MP. Recently, the TMV MP was shown to be associated with the ER in *Nicotiana benthamiana* leaves and BY-2 protoplasts either infected with TMV derivatives encoding the MP as an MP-GFP fusion, or transfected with an MP-GFP gene construct (Heinlein et al. 1998; Reichel and Beachy 1998). The association of the AIMV MP with the ER reported here is

therefore not a unique characteristic of one specific viral MP. Instead, the association may be part of a general mechanism by which viral MPs function in promoting viral movement from an initially infected cell to the surrounding uninfected cells. In fact, we have shown in this study that the MP-GFP expressed in a primarily transfected cell is able to move to neighboring cells. Taking into consideration the close relationship between the ER and the MP-GFP, the dynamic activity of the MP is most likely derived from its association with the ER.

It is generally accepted that Pd are the intercellular route for plant virus cell-to-cell movement (Robards and Lucas 1990; Lucas and Gilbertson 1994; Waigmann and Zambryski 1995; Blackman et al. 1998). It has also been demonstrated that Pd gating allows the viral MP to move through the cell wall (Wolf et al. 1989; Derrick et al. 1992; Fujiwara et al. 1993; Poirson et al. 1993; Vaquero et al. 1994; Ding et al. 1995; Angell et al. 1996). The desmotubule in Pd was previously postulated to form a membrane channel composed of strands of the ER threading through the cell wall (Lopez-Saez et al. 1966; Overall et al. 1982). Two types of Pd have been classified (Lucas et al. 1993). The primary Pd are characterized as containing a single channel commonly seen after cell division, while the secondary Pd are characterized as containing branched structures that have undergone extensive modification (Franceschi and Lucas 1982; Hepler 1982; Esau and Thorsch 1985; Ding et al. 1992a, 1992b). The regular distribution of fluorescent strands or spots (Figs. 1B, 2B, and 4A) in the cell wall of MP-GFP-expressing cells suggests that the MP-GFP might be transported to neighboring cells through Pd. This is further supported by the observation of the continuity of the MP-GFP-labeled filamentous structure between neighboring cells and the co-localization of the MP-GFP with Pd visualized by CLSM (Fig. 5). Consistent with these results, the AIMV MP was previously detected in a cell wall fraction prepared from infected tobacco leaves (Godefroy-Colburn et al. 1986). It is therefore likely that the transiently expressed MP-GFP in plant cells may behave similarly to that in virus-infected leaves.

The physical continuity of the ER membrane between adjacent cells suggests a potential pathway for the movement of

Fig. 8. Distribution of movement protein–green fluorescent protein (MP-GFP) in Sf9 cells infected with BacMPGFP and co-localization of the MP-GFP with the endoplasmic reticulum (ER) in insect cells. **A**, Lateral cross section showing accumulation of the MP-GFP in the cortex region of an Sf9 cell at 4 days post infection (pi). **B**, Immunofluorescent staining of ER membranes in same cell shown in **A** with anti-BiP and rhodamine B conjugated IgG. **C**, Overlaying of **A** and **B**, showing co-alignment of the MP-GFP and the ER in an Sf9 cell. Images were taken by confocal laser scanning microscopy. Bar: 10 μ m.

lipophilic molecules and possibly membrane-associated proteins (Grabski et al. 1993). With the use of confocal microscopy and FRAP (fluorescence recovery after photobleach), lipids and phospholipids were found to be able to traverse the cell wall barrier by ER membrane-mediated diffusion (Grabski et al. 1993). The MP-GFP behaved as an integral ER membrane protein, which suggests that intracellular and intercellular transport of the MP-GFP might occur via ER membrane-mediated trafficking. However, under physiological conditions Pd only allow transport of molecules smaller than 1 kDa. How the MP-GFP, with a molecular mass of 59 kDa, passes through Pd is not known. It is likely that virus-encoded MPs might be able to target to, and interact with, the substructural components of Pd, resulting in the dilation of the micro-channels. This would then allow spread of the MP into a neighboring cell (Mezitt and Lucas 1996). Evidence presented in this report would lend support to this hypothesis. Investigation into the mechanism by which the MP modifies Pd and identification of the active Pd component(s) would significantly increase our understanding of plant virus cell-to-cell spread as well as plant cell communication pathways.

MATERIALS AND METHODS

Cells and plasmids.

The epidermis of onion bulb scales was prepared by peeling the inner epidermis from fresh onion bulbs. The BY-2 suspension cells were maintained as described by Banjoko and Trelease (1995). The *Spodoptera frugiperda* (Sf9) lepidopteran cells were maintained in SF900 culture medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% NBS (new-born bovine serum) at 28°C in the dark.

The MP-GFP and GFP expression vectors pE6113MPGFP and pE6113GFP were constructed as described previously (Zheng et al. 1997).

Construction of recombinant baculovirus (BacMPGFP).

The MP-GFP gene was constructed under the control of the baculovirus polyhedrin promoter by replacing the polyhedrin gene with the MP-GFP gene, using the Baculovirus Expression System kit according to the instructions of the manufacturer (Clontech Laboratories, Palo Alto, CA). Briefly, a DNA fragment encoding the MP-GFP fusion protein was derived from pE6113MPGFP by digestion with restriction enzymes *Xba*I and *Sac*I. The *Xba*I-*Sac*I fragment was ligated into the *Xba*I/*Sac*I linearized transfer vector (pBacAK8), giving the recombinant transfer vector, pBacAK8MPGFP, which was transfected into Sf9 cells along with Bsu361-digested BacPAK6 viral DNA. The target gene was recombined with the viral DNA in vivo to rescue the viral DNA and assemble the recombinant virus BacMPGFP. The recombinant virus particles were further amplified by infection of Sf9 cells. The BacGFP recombinant virus was generated by the same method.

Antibodies and reagents.

Polyclonal anti-GFP antibody was obtained from Promega (Madison, WI), anti-rabbit IgG-rhodamine was obtained from Roche Molecular Biochemicals (Mannheim, Germany), and rabbit polyclonal anti-BiP (GRP78) antibody was obtained from Affinity Bioreagents (Golden, CO). Hexyl ester of rhodamine B was obtained from Molecular Probes (Eugene, OR).

Extraction of proteins from membranes.

Sf9 cells infected with BacMPGFP were collected by centrifugation at $2,500 \times g$ for 5 min. The cells were homogenized in pre-chilled homogenization buffer (0.4 M sucrose, 1 mM dithiothreitol [DTT], 0.1 mM EDTA, 3 mM $MgCl_2$, 40 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid [HEPES]-NaOH pH 7.5, 10 mM KCl). The P30 and S30 fractions were separated by centrifugation of homogenized Sf9 cells at $30,000 \times g$ for 1 h. The supernatants were collected and the pellets resuspended in homogenization buffer (equal volume to the decanted supernatant).

The MP-GFP fusion protein was extracted from the pool of fractions by the method described by Subramaniam et al. (1995). Briefly, aliquots of the pellets were incubated with either 1 M NaCl, 1 M KCl, 100 mM $NaHCO_3$ (pH11), 150 mM Na_2CO_3 , (pH 11.5), 1% NP-40, or 1% Triton-100 in 10 mM Tris pH 7.4 for 1 h on ice. Samples were then centrifuged at $150,000 \times g$ for 1 h, and polypeptides in the supernatants and the pellets, respectively, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot).

SDS-PAGE and Western blot.

Samples were boiled with an equal amount of 2× SDS gel loading buffer (Promega) for 2 min then subjected to 12% SDS-PAGE. Proteins separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane in transfer buffer (0.5 mM Tris-HCl, 0.2 M glycine, 20% methanol) at 60 V for 4 h. The membrane was blocked in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 3% nonfat milk for 2 h at room temperature then blotted with first antibody in TBST buffer for 1 h. After three washes with TBST, the membrane was transferred to TBST buffer containing 1:5,000 diluted anti-IgG AP (Promega) for 30 min with gentle agitation. The membrane was washed and then subjected to a color development reaction with the Proto Blot Western Blot AP System (Promega).

Transfection of plant cells by particle bombardment.

Onion epidermis was mounted onto glass slides. For each DNA construct, 2 µg of DNA was mixed with 25 µl of 0.1 M spermidine, 50 µl of 2.5 M $CaCl_2$ and 50 µl of gold microcarrier (20 µg/ml in water), vortexed vigorously for 3 min, and centrifuged at 10,000 rpm (Eppendorf centrifuge 5415C; Hamburg, Germany) in a microcentrifuge for 10 s. The pellet was washed once with 250 µl of ethanol then re-suspended in 30 µl of ethanol. The suspension was loaded onto one or two disks (1,100 to 1,300 lb/in² kit; Bio-Rad, Hercules, CA) and air dried. The DNA-gold particles were bombarded into cells at a pressure of 1,100 lb/in² with the Helios gene gun system (Model PDS-1000/He Biolistic particle delivery system; Bio-Rad). The transfected cells were kept alive by submerging in Murashige-Skoog (MS) medium.

Transfection of tobacco epidermal cells was carried out as described by Itaya et al (1997).

Staining of the ER and fluorescence microscopy.

The ER of tobacco BY-21 cells, onion epidermal cells, and Sf-9 insect cells was stained with the fluorescent probe hexyl ester of rhodamine B at a concentration of 1 mg/ml, as described by Grabski et al (1993).

Fluorescence microscopy was carried out with an inverted epifluorescence microscope (Olympus Ix70, Tokyo). A DM500 dichroic mirror/BP450-480 exciter filter/BA515 emission filter set was used. Photographs were taken with the PM-30 automatic photomicrographic system (Olympus). CLSM was performed with an MRC1024 CLSM (Bio-Rad Microscopy Division, Hemel Hempstead Herts, U.K.) with a krypton/argon laser with three lines 488/568/647nm.

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