All five of the proteins encoded by the three genomic RNAs of *Cucumber mosaic virus* (CMV) affect the movement of CMV (reviewed by Palukaitis & García-Arenal, 2003). However, the 3a protein encoded by CMV RNA 3 is considered to be the primary movement protein (MP) (Canto et al., 1997; Kaplan et al., 1995; Nagano et al., 1997). Various observations demonstrated a role for the CMV 3a protein in movement, e.g. the 3a protein bound single-stranded RNA cooperatively in vitro (Kim et al., 2004; Li & Palukaitis, 1996), potentiated the movement of itself, RNA and fluorescent dextran through plasmodesmata (Ding et al., 1995) and localized to plasmodesmata between various cell types and to parietal sieve elements in the phloem (Blackman et al., 1998). The 3a protein fused to green fluorescent protein (GFP) was also able to form tubules extending from the surface of protoplasts (Canto & Palukaitis, 1999a).

Natural and artificial mutants of the 3a protein have been shown to affect CMV movement in specific hosts or tissues (Canto & Palukaitis, 1999a, b; Kaplan et al., 1997; Li et al., 2001; Takeshita et al., 2001) and mutants of the 3a protein also have been identified that affect cell-to-cell versus long-distance movement (Li et al., 2001). Various 3a protein mutants generated by alanine-scanning mutagenesis (Table 1; Fig. 1a) were shown to be deficient in various functions. Mutants designated M4–M7 were unable to promote cell-to-cell movement, although CMV containing mutants M4, M5 and M6 could be complemented for cell-to-cell and long-distance movement in transgenic tobacco plants expressing the 3a protein (Li et al., 2001). The movement-defective mutant designated M5 was also shown to be deficient for the gating of plasmodesmata, with no cell-to-cell movement of itself, RNA or fluorescent dextran (Ding et al., 1995). The mutant designated M8 was unable to induce tubule formation in infected *Nicotiana benthamiana* protoplasts or promote the cell-to-cell movement of CMV in the epidermal cells of either *Nicotiana tabacum* (Canto & Palukaitis, 1999a) or *Chenopodium amaranticolor* (Canto & Palukaitis, 1999b). However, whether the various other mutations affected the ability of the CMV 3a protein to target plasmodesmata in epidermal cells and to assemble in protruding tubular structures in protoplasts has not been reported. Such might be expected, as mutants of the MPs of *Alfalfa mosaic virus* (AMV), *Cowpea mosaic virus* (CPMV) and *Tobacco mosaic virus* (TMV), generated by alanine scanning, have shown differences in their localization patterns (Huang et al., 2001; Kahn et al., 1998; Pouwels et al., 2003). To examine the importance of subcellular localization vis-à-vis the proper function of the CMV MP, we have examined the subcellular distribution of the various mutant 3a proteins fused to GFP in epidermal cells of two host species (*N. benthamiana* and *N. tabacum*). We also have examined the localization of the various GFP-tagged MP mutants in infected *N. benthamiana* protoplasts derived from mesophyll cells.

The wild-type 3a protein per se, as well as this protein fused to GFP at its C terminus, both localized to plasmodesmata, as determined previously by both electron microscopic immunolocalization and confocal laser-scanning microscopy (Blackman et al., 1998). Therefore, confocal microscopy was
Table 1. Infectivity and subcellular distribution of the wild-type and nine alanine-scanning mutants of the MP of CMV fused to GFP

<table>
<thead>
<tr>
<th>Virus (mutated position)*</th>
<th>Infectivity†</th>
<th>Distribution‡</th>
<th>Tubule quantification§</th>
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</thead>
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<tr>
<td></td>
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<td>Epidermal cells</td>
<td>Mesophyll protoplasts</td>
</tr>
<tr>
<td>Wild-type</td>
<td>+</td>
<td>Pd</td>
<td>Tubules, ps Ag</td>
</tr>
<tr>
<td>M1 (R8, T9)</td>
<td>+</td>
<td>Pd</td>
<td>Tubules, ps Ag</td>
</tr>
<tr>
<td>M2 (E38, D40)</td>
<td>+</td>
<td>Pd</td>
<td>Tubules, ps Ag</td>
</tr>
<tr>
<td>M3 (Y75, D76)</td>
<td>+</td>
<td>Pd, Pm</td>
<td>Tubules, ps Ag</td>
</tr>
<tr>
<td>M4 (R97, T98)</td>
<td>−</td>
<td>C, N, (c Ag)</td>
<td>C, N, (Ag)</td>
</tr>
<tr>
<td>M5 (Y144, D145)</td>
<td>−</td>
<td>C, N, (c Ag)</td>
<td>C, N, (Ag)</td>
</tr>
<tr>
<td>M6 (R156, F158)</td>
<td>−</td>
<td>C, N, (c Ag)</td>
<td>C, N (Ag)</td>
</tr>
<tr>
<td>M7 (N191, Y192)</td>
<td>−</td>
<td>C, (Pm?)</td>
<td>C, Pm</td>
</tr>
<tr>
<td>M8 (D20, D21)</td>
<td>±</td>
<td>C, N, (c Ag)</td>
<td>C, Pm</td>
</tr>
<tr>
<td>M9 (P60)</td>
<td>±</td>
<td>C, N, Pm, (c Ag)</td>
<td>C, Pm, N</td>
</tr>
</tbody>
</table>

*The mutants contain alterations to alanine in the 3a protein sequence at the positions indicated.
†Infectivity of the wild-type and mutant MPs as determined on six systemic hosts of CMV and two local lesion hosts (Li et al., 2001). +, Infectious on all hosts tested; −, no detectable movement on any hosts tested; ±, infectious on all systemic hosts at room temperature, but either not infectious on local lesion hosts and not able to move between epidermal cells (M8), or temperature-sensitive for long-distance movement in tobacco (M9).
‡Abbreviations: C, cytoplasm; c Ag, cytoplasmic aggregates; N, nucleus; Pd, plasmodesmata; Pm, plasma membrane; ps Ag, punctate surface aggregates.
§No. protoplasts with filaments/total infected cells counted.

used here to examine the distribution of the mutant 3a proteins fused to GFP (Fig. 1), as was done previously for the wild-type 3a protein and the mutant M8 (Canto & Palukaitis, 1999a; Canto et al., 1997). PCR mutagenesis was used to fuse sequences encoding the GFP to those encoding the various mutant 3a proteins. The fusion proteins were expressed from the corresponding mutated CMV RNAs 3, following inoculation of plants with RNA transcripts of CMV RNAs 1, 2 and the mutated RNAs 3, all as described previously (Canto & Palukaitis, 1999a; Canto et al., 1997; Li et al., 2001). The various mutant 3a proteins were shown previously to accumulate to similar levels in infected N. tabacum protoplasts (Li et al., 2001).

In inoculated N. tabacum and N. benthamiana plants, considerable variation was found between mutants in the number of fluorescent foci detected by confocal microscopy between 2 and 5 days post-inoculation (p.i.). In most cases, fluorescence levels were very weak and many infected foci may have escaped detection (data not shown). Nevertheless, the patterns of distribution observed in inoculated epidermal cells were consistent from focus to focus and from plant to plant for a given mutant and, in general, were similar in N. tabacum (Fig. 1b) and N. benthamiana (Fig. 1c).

In epidermal cells, those mutants that were functional for movement (M1, M2, M3, M8 and M9) showed two general types of distribution of the MP–GFP fusions (Table 1): either localizing to plasmodesmata (M1, M2 and in part M3), often spreading to plasmodesmata in cells neighbouring the initially infected cell (for mutants M1 and M2, as for the wild-type 3a–GFP fusions) (Figs 1b and c, arrows) or partitioning between the cell periphery and elsewhere in the cells (M8 and M9). In the case of the M8 and M9 MP–GFP fusions, fluorescence was confined to the initially infected cells, in the form of small aggregates, with M8 distributed uniformly throughout the cytoplasm as well as the nucleus, and M9 distributed in the cytoplasm in a more granular fashion as well as in the nucleus (Figs 1b and c). It was not possible to ascertain whether the fluorescence distributed along the periphery of the cell was also associated with plasmodesmata, although mutant M9 did show several punctate bodies that suggested as such (Figs 1b and c). The absence of an association with plasmodesmata for mutant M8 was not surprising, as this mutant was shown previously to be unable to promote the movement of CMV between epidermal cells (Canto & Palukaitis, 1999a, b).

For those mutants that were dysfunctional for movement (M4, M5, M6 and M7), fluorescence was confined to the initially infected cells, both in N. tabacum and in N. benthamiana. The fluorescence pattern of mutants M4, M5 and M6 showed some similarities in their distribution, in that the MP–GFP fusions were not localized to plasmodesmata, but showed partitioning between the nucleus and cytoplasm, including transvacuolar strands (Fig. 1b). Mutant M4 seemed to produce mostly smaller, uniformly sized...
fluorescent aggregates than either mutants M5 or M6, both of which produced quite large fluorescent inclusion bodies, although there was some variability between samples. The M7 MP–GFP fusion showed high levels of fluorescence distributed throughout the oppressed cytoplasm (Fig. 1b). The dysfunctional mutant MPs showed a distribution of fluorescence in *N. benthamiana* (Fig. 1c) similar to that in *N. tabacum* (Fig. 1b).

Unlike the situation with TMV (Boyko *et al.*, 2000; Heinlein *et al.*, 1995, 1998; McLean *et al.*, 1995), neither the wild-type CMV MP nor the dysfunctional mutants appeared to be associated with the microtubules. To verify this, the CMV 3a MP was fused to the monomer red fluorescent protein and expressed after agroinfiltration into either transgenic *N. benthamiana* expressing an α-tubulin–GFP fusion (Gillespie *et al.*, 2002) or *N. benthamiana* plants co-agroinfiltrated

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**Fig. 1.** Subcellular distribution of CMV wild-type (WT) and mutant (M1–M9) 3a–GFP fusions. (a) Schematic map of mutations M1–M9 in the 3a protein of CMV. Mutants listed above the rectangle are competent for movement, whereas those listed below the rectangle are dysfunctional for movement; those mutants both above and below the rectangle are conditionally functional for movement. (b, c) Confocal-microscopy images of WT and mutant 3a–GFP fusions in infected epidermal cells of (b) *N. tabacum* and (c) *N. benthamiana* 3 days p.i. Arrows in (b) and (c) indicate plasmodesmatal targeting occurring in cells adjacent to the initially inoculated one. Bars, 100 μm.
with a plasmid expressing the α-tubulin–GFP fusion (Ueda et al., 1999). Both approaches gave similar results. Neither the wild-type nor two mutant MPs (M4 or M8) showed co-localization with α-tubulin–GFP (see Supplementary Figure in JGV Online). Given that the MPs of several other viruses also have been shown not to require microtubules for tubule formation (Huang et al., 2000, 2001; Laporte et al., 2003; Pouwels et al., 2002), this is not a unique situation. Moreover, the interaction of the TMV MP with microtubules has been shown to be associated with a degradation pathway (Gillespie et al., 2002; Kragler et al., 2003) and, unlike the TMV MP, the CMV 3a protein did not interact with a microtubule-associated protein designated MPB2C (Kragler et al., 2003).

The distribution of the MP mutants within epidermal cells was compared with that in mesophyll protoplasts (Table 1) by preparing N. benthamiana protoplasts and infecting them with CMV RNA transcripts expressing the various mutant MP–GFP fusions, as described previously for the wild-type 3a MP and mutant M8 (Canto & Palukaitis, 1999a). The protoplasts were analysed by fluorescence microscopy at two time points: 27 and 51 h p.i. (Fig. 2). In different experiments, the percentage of infected protoplasts

![Fig. 2. Subcellular distribution of CMV wild-type (WT) and mutant (M1–M9) 3a–GFP fusions in infected mesophyll protoplasts of N. benthamiana 27 and 51 h after electroporation. Arrows point to distinct protruding filament structures. Bar, 100 μm.](image-url)
varied between 12.5 and 66%, as measured by fluorescence (data not shown). The distribution of the MP–GFP fusions in the infected protoplasts varied with both the sample time and the mutant. As described previously (Canto & Palukaitis, 1999a), the wild-type 3a–GFP showed the presence of aggregates of MP on the surface of the protoplasts, forming tubules. The number of aggregates and tubules was greater at 51 h p.i. than at 27 h p.i. (Fig. 2 and data not shown). The tubules were very fragile and could break off during preparation of the samples for viewing. Only the MP mutants M1, M2 and M3, all of which were functional for virus movement in all hosts tested, showed the presence of fluorescent tubules on the surface of the infected protoplasts (Fig. 2; Table 1). Interestingly, the tubules were more abundant in the case of mutants M1 and M2 than in the wild-type 3a–GFP fusions (Table 1).

The MP mutants that were dysfunctional for movement (M4, M5, M6 and M7) all showed some similar features, including the absence of distinct tubules (Table 1). M4, M5 and M6 all showed the presence of small and/or large aggregates in the cytoplasm and association of fluorescence with transvacuolar strands (Fig. 2). M7 showed some features similar to these, but not large inclusions, and M7 also showed higher fluorescence along the periphery of the protoplasts. None of the mutants M4, M5, M6 or M7 was able to form the discrete, punctate spots that were observed on the surface of protoplasts infected with either the wild-type 3a–GFP fusion or fusions involving the other mutants that could form tubules (M1, M2 and M3). This suggests that the punctate spots are precursors of the tubules, as has been concluded for tubules forming on the surface of protoplasts infected with several other viruses [AMV, *Cauliflower mosaic virus* (CaMV) and CPMV] expressing MP–GFP fusions (Heinlein et al., 1998; Huang et al., 2000; Pouwels et al., 2002, 2003).

The MP mutants M8 and M9, both of which are conditionally functional for movement, also did not show the formation of tubules on the surface of infected protoplasts (Fig. 2; Table 1; Canto & Palukaitis, 1999a). Rather, M8 showed MP distributed throughout the cell, although somewhat more concentrated along the periphery, whereas M9 showed MP distributed mostly along the periphery, i.e. presumably associated with the plasma membrane and with some isolated spots on the surface (Fig. 2). The MPs of both M8 and M9 also showed some association of fluorescence with the nucleus, as was observed for the dysfunctional mutants (Fig. 2; Table 1).

The role of the CMV 3a protein in tubule formation remains unclear, as this property was shown not to be essential for CMV movement (Canto & Palukaitis, 1999a), in contrast to the situation with AMV (Huang et al., 2001). Moreover, no tubule-like structures could be detected between cells in CMV-infected plants (Blackman et al., 1998). By contrast, the MP of the ilarvirus *Olive latent virus* 2, which, like CMV and AMV, is also in the family *Bromoviridae*, did form such tubules containing virus particles (Grieco et al., 1999). The MPs of both CPMV and CaMV form tubules between infected cells containing virus particles, through which it is believed that cell-to-cell movement occurs (Kasteel et al., 1996; Linstead et al., 1988; van Lent et al., 1990). However, the MPs of these viruses also were able to bind RNA *in vitro* (Carvalho et al., 2004; Citovsky et al., 1991), as were the MPs of CMV (Li & Palukaitis, 1996) and AMV (Schoumacher et al., 1992). Thus, it is conceivable that many viruses retain elements of both major movement pathways, whilst only one pathway is used for a given virus. That these bifunctional elements are still conserved may be a consequence of other host–virus interactions associated with aspects of virus movement that are common to the two pathways, which have yet to be determined.

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**References**


