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The Cowpea Mosaic Virus M RNA-Encoded 48-Kilodalton Protein Is Responsible for Induction of Tubular Structures in Protoplasts

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Tubular structures extending from plasmodesmata in cowpea mosaic virus (CPMV)-infected tissue have been implicated to play an important role in cell-to-cell movement of this virus. Using a cauliflower mosaic virus 35S promoter-based transient expression vector, we show that expression of only the CPMV M RNA-encoded 48-kDa protein (48K protein) in cowpea protoplasts is sufficient to induce these structures. Strikingly, expression of the 48K protein in protoplasts from a number of nonhost plant species, such as barley, *Arabidopsis thaliana*, and carrot, also resulted in tubular structure formation. Thus, it is not likely that the viral 48K protein, though playing a key role in cell-to-cell movement of CPMV, has a role in determining the host range of CPMV.

Cowpea mosaic comovirus (CPMV) has divided its genetic information over two plus-strand RNA molecules (for a review, see reference 2). The larger B RNA codes for proteins involved in replication, whereas the M RNA-encoded proteins are involved in encapsidation and cell-to-cell movement. M RNA is translated into two overlapping carboxy coterminal proteins with sizes of 105 and 95 kDa (105K and 95K proteins) as a result of translational initiation at two separate AUG codons at nucleotide positions 161 and 512 (4, 7, 18). Processing by the B RNA-encoded protease results in the 58K and 48K proteins, with overlapping sequences, and the capsid proteins VP37 and VP23 (3). Both genetic analysis and electron microscopy suggest that CPMV moves from cell to cell as whole particles with the aid of tubular structures that penetrate through presumably modified plasmodesmata into the neighboring cell (15, 16, 20). Whether existing plasmodesmata are modified or whether new connections between cells are induced by the virus is still an open question.

The tubular structures, typical for CPMV-infected plant tissue, are also formed in isolated cowpea protoplasts lacking cell walls and plasmodesmata (15). Immunogold-labeling and immunofluorescence detection experiments revealed that the tubular structures contain the 58K and/or 48K protein (15, 16). The antiserum used in these studies was raised against a synthetic peptide with 30 residues corresponding to the common C terminus of the overlapping 58K and 48K proteins and thus did not allow discrimination between these two proteins (16, 19). Further analysis of the individual functions of the 58K and 48K proteins and the capsid proteins in tubule formation was assessed by using a mutant virus which failed to produce the capsid proteins. This mutant is still capable of inducing tubules, devoid of virus particles, showing that the capsid proteins have no role in the morphogenesis of these structures (6). On the other hand, mutants which fail to produce the 48K protein or which produce a truncated 48K protein are no longer able to induce tubules, suggesting that at least the 48K protein is essential for tubule formation (6). These studies, using CPMV M RNA mutants, did not permit conclusions about the separate role of the 58K protein in this process.

Employing a transient expression system based on the 35S promoter of cauliflower mosaic virus (11), we now show that synthesis of the 48K protein in the absence of the viral replication process is sufficient to induce tubules in cowpea protoplasts and even in protoplasts derived from plant species which are not hosts for CPMV.

All DNA manipulations were done essentially as described previously (9). Plasmid pMon999 (a generous gift from C. Hemenway, Monsanto Company) contains a multiple cloning site between an enhanced 35S promoter and the nopaline synthase (*nos*) terminator. The construction of pTMB200, which contains the 200K open reading frame of B RNA inserted in pMon999, has been described previously (11). The construction of pTMΔAUG0, which contains a newly created *Sst*II site at position 114 in the M cDNA, will be described in more detail somewhere else. Construction of pTM58SΔ3, which contains a stop codon at the end of the 58K open reading frame and lacks the 1537 to 2100 fragment of the M cDNA, has been described previously (6). Plasmid pMM58/48 consists of the *Xba*I-*Sst*II linker fragment of pBS (Stratagene), the 70-bp *Sst*II-*Bgl*II fragment of pTMΔAUG0, and the 1.7-kb *Bgl*II-*Sst*I fragment of pM58SΔ3 in pMon999 which was linearized with *Xba*I and *Sst*I (see Fig. 1). Plasmid pMM48 is constructed by inserting the 1.7-kb *Bgl*II-*Sst*I fragment of pTM58SΔ3 into pMon999 linearized with the same enzymes.

Cowpea and pea protoplasts (11); tomato protoplasts (13); *Arabidopsis thaliana*, carrot, and cactus (Saguaro) protoplasts (1); and barley protoplasts (5) were transfected with 1 μg of CPMV RNA or 10 μg of DNA per 10^6 protoplasts with polyethylene glycol (11). Protoplasts were incubated in medium under continuous illumination (8).

To study the separate involvement of the 58K and 48K proteins in the induction of tubular structures, the coding sequence of the 58K and 48K proteins and that of the 48K protein alone were inserted between the enhanced 35S

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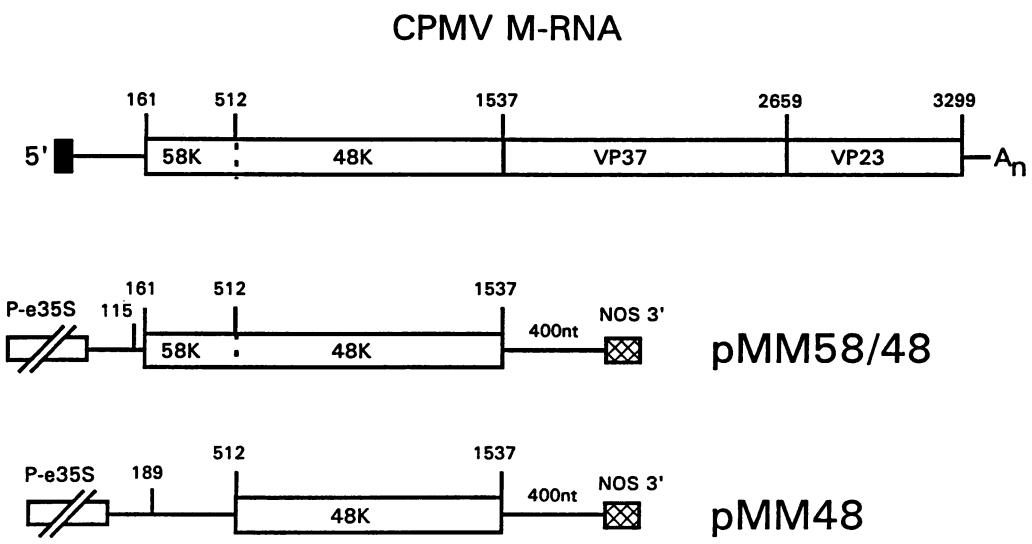

FIG. 1. Schematic diagram of CPMV M RNA and DNA constructs used in this study. Open bars, open reading frames; closed bar, VPg; A_n, poly(A) tail; P-e35S, enhanced 35S promoter of cauliflower mosaic virus; hatched box, *nos* terminator sequence. The relevant nucleotide positions of M RNA and M cDNA are shown above each diagram.

promoter and *nos* terminator in vector pMon999, resulting in the constructs pMM58/48 and pMM48, respectively (Fig. 1). Transcripts from pMM58/48 are expected to possess a 5′ noncoding region consisting of sequences from the multiple cloning site of pMon999 and from CPMV M RNA from positions 115 to 161 (17) (Fig. 1) and should be able to direct the synthesis of both the 58K and 48K proteins. Transcripts from pMM48 are expected to possess a 5′ noncoding region consisting of sequences from the multiple cloning site of pMon999 and from CPMV M RNA from positions 189 to 512 (Fig. 1). These transcripts should be able to direct the synthesis of only the 48K protein since the AUG codon at the beginning of the open reading frame of the 48K protein is the first AUG codon present in the transcripts (17). Cowpea protoplasts were transfected with these DNA constructs and 17 h later were analyzed for their protein content by Western blotting (immunoblotting) with the anti-58K/48K serum (12, 19). Neither the 58K protein nor the 48K protein was detectable in extracts of protoplasts transfected with pMM58/48 (Fig. 2, lanes 1, 2, and 6). However, in extracts of protoplasts transfected with pMM48, a 48K protein which comigrated with the 48K protein present in extracts of protoplasts inoculated with wild-type CPMV RNA was present (Fig. 2, lanes 5 and 7). This protein migrated slightly faster than the 48K protein present in the membrane fraction of CPMV-infected plant material (Fig. 2, lane 9). At the moment, no plausible explanation can be given for this difference in mobility. Several bands present in immunoblots of protein extracts from pMM48- and CPMV RNA-transfected cells, migrating faster than the 48K protein, possibly represent degradation products of this 48K protein. Faint but distinct immunoreactive bands corresponding to protein sizes of approximately 90K and 130K may represent dimers and trimers of the 48K protein (19).

At 17 h after transfection with pMM58/48 and pMM48, protoplasts were prepared for immunofluorescence microscopy with the anti-58K/48K serum (15). Approximately 5% of the protoplasts transfected with pMM48 showed tubular structures extending from their surfaces as observed for protoplasts inoculated with CPMV RNA (Fig. 3A and C).

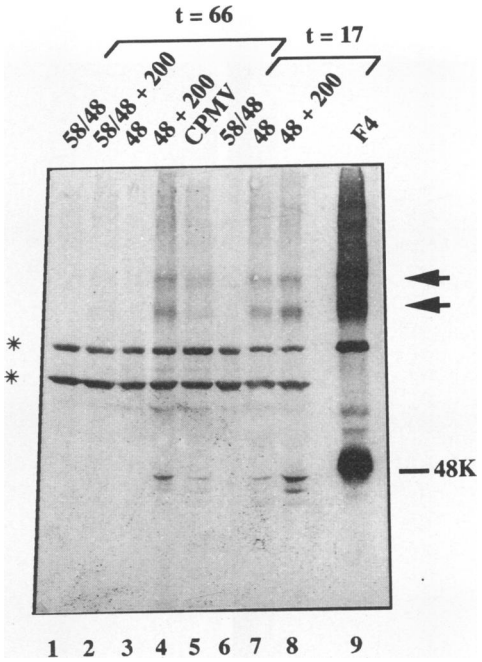

FIG. 2. Detection of the CPMV M RNA-specific 48K protein in transfected protoplasts. Extracts of protoplasts isolated 17 h (lanes 6 to 8) and 66 h (lanes 1 to 5) after transfection with pMM58/48 (lanes 1 and 6), pMM58/48 + pMB200 (lane 2), pMM48 (lanes 3 and 7), pMM48 + pMB200 (lanes 4 and 8), and CPMV RNA (lane 5) were separated on a sodium dodecyl sulfate–10% polyacrylamide gel and blotted onto nitrocellulose. Lane 9 contained a membrane fraction of CPMV-infected cowpea leaves (F4). The Western blot was incubated with anti-58K/48K serum and goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate. Asterisks denote nonspecific reacting proteins. The arrows denote putative multimers of the 48K protein.

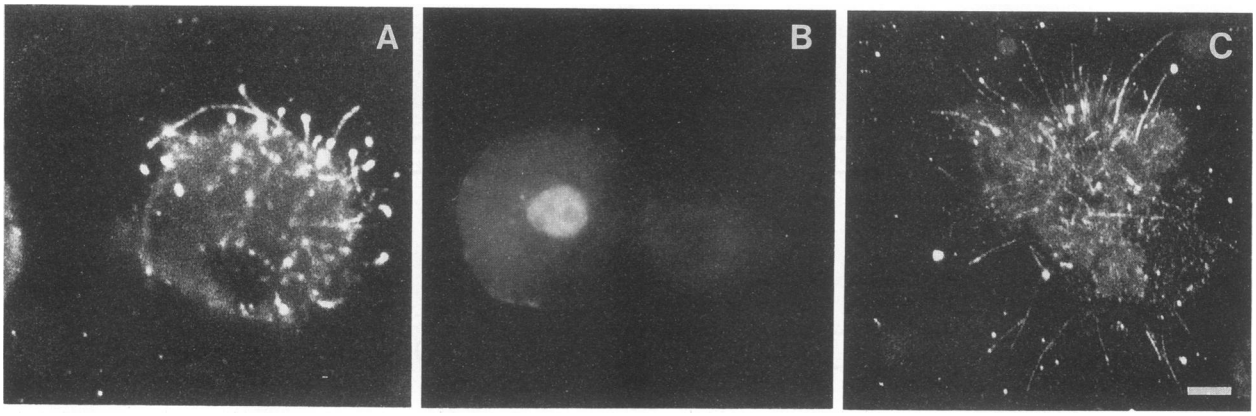

FIG. 3. Immunofluorescent images of transfected protoplasts treated with anti-58K/48K serum. The confocal scanning laser microscope images (A and C) represent 1-μm-thick optical sections. Shown are cowpea protoplasts transfected with CPMV RNA, with tubules extending into the medium (A); with pMM58/48, showing distinct staining of the nucleus (B); or with pMM48, with numerous tubules extending into the medium (C). Bar, 5 μm.

Furthermore, diffuse staining of the cytoplasm was observed in these protoplasts. Although immunoblotting did not reveal the expression of either 58K or 48K proteins, about 1% of the protoplasts transfected with pMM58/48 showed tubules extending from their surfaces (data not shown). Furthermore, tubules were observed on the surface of the microscope slide, obviously remaining there after detachment of protoplasts during the fluorescence-staining procedure. Additionally, and different from pMM48-transfected protoplasts, approximately 10% of pMM58/48-transfected protoplasts showed staining of the nucleus (Fig. 3B), comparable to CPMV RNA-infected protoplasts (15). This phenomenon implies that it is mainly the 58K protein that associates with the nucleus. A similar conclusion was drawn from experi-

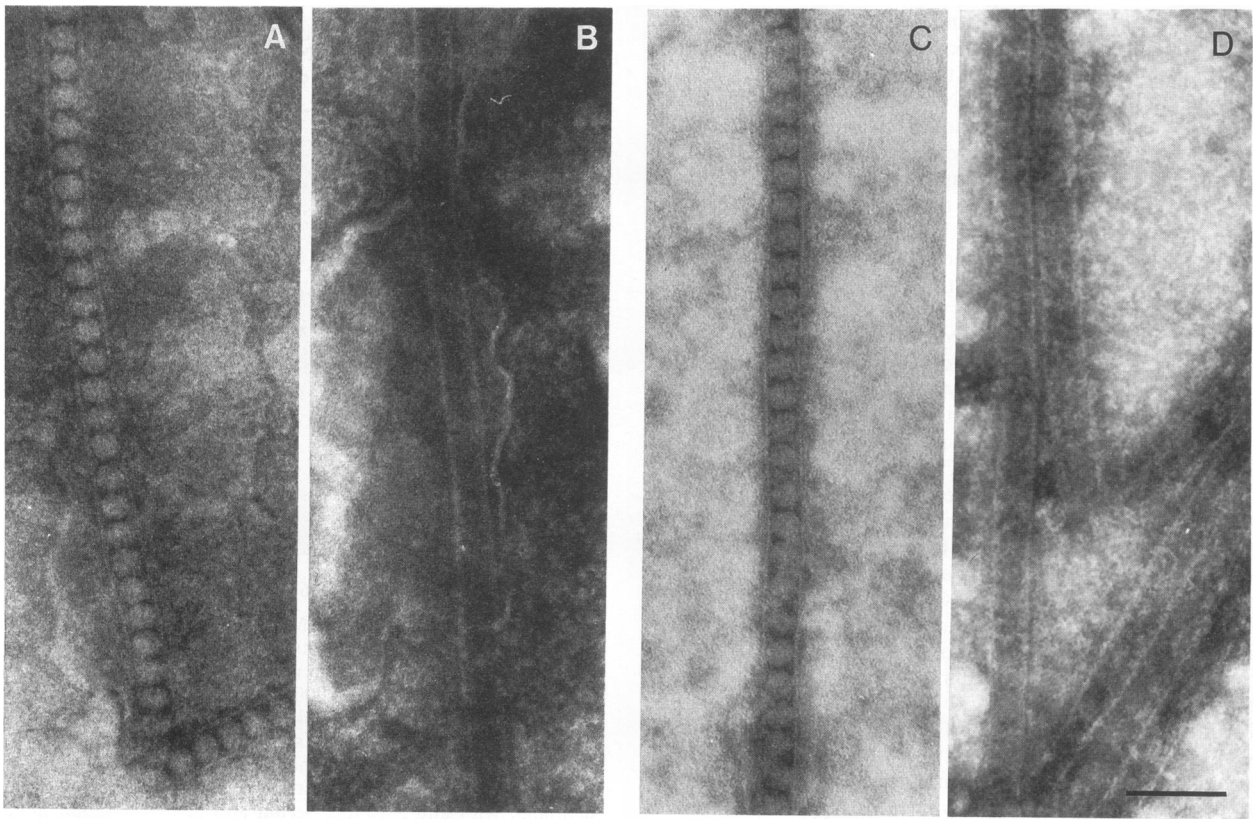

FIG. 4. Electron micrographs of negatively stained preparations of transfected protoplasts. (A) Tubules from cowpea protoplasts transfected with CPMV RNA; (B) empty tubule from cowpea protoplasts transfected with pMM48; (C) tubule from *Arabidopsis* protoplasts transfected with CPMV RNA; (D) empty tubules from *Arabidopsis* protoplasts transfected with pMM48. Bar, 100 nm.

TABLE 1. Induction of tubules in protoplasts of various plant species transfected with CPMV RNA and pMM48

Protoplast	CPMV RNA		pMM48
	Infection (%)[a]	Tubules[b]	Tubules[b]
Cowpea	90	++	++
Cowpea TVu470	90	++	ND[c]
Pea	90	++	++
Tomato	40	++	++
A. thaliana	10	++	++
Barley	3	+	+
Carrot	0.5	+	+
Cactus	ND	ND	++

[a] Determined by immunofluorescence with anti-CPMV serum and expressed as the percentage of living cells showing a positive reaction.

[b] Determined by immunofluorescence with anti-58K/48K serum and electron microscopy. ++, many tubules (>50); +, only a few tubules (<10) (observed by immunofluorescence staining of approximately 3×10^3 protoplasts).

[c] ND, not determined.

ments with a mutant virus that failed to produce the 48K protein (6). Whether the presence of the 58K protein in the nucleus is required for its functioning is not clear at the moment.

Negative-staining electron microscopy (15) revealed, as expected, that the tubules induced in both pMM48- and pMM58/48-transfected protoplasts did not contain virus particles but were empty (Fig. 4B). These tubules have an appearance (e.g., diameter and length distribution) similar to that of tubules produced by wild-type virus (Fig. 4A and B) and a CPMV M RNA mutant which fails to produce capsid proteins (6). Obviously, all these results indicate that expression of only the 48K protein, even in the absence of the virus multiplication process, is sufficient for tubule formation in cowpea protoplasts.

At later times after transfection, the number of tubules and the fluorescence signal in protoplasts diminished, and at 66 h, fluorescence staining in the nucleus had completely disappeared. Remarkably, when either pMM58/48 or pMM48 was cotransfected with pMB200, which expresses the complete B RNA-coding region (11), the numbers of fluorescent cells and tubules were higher (approximately 2 and 10%, respectively). This observation was confirmed by Western blot analysis, revealing larger amounts of 48K protein in protoplasts when B RNA-encoded proteins were present (Fig. 2). Currently, no explanation for this enhancing effect of the B proteins can be given.

To test whether the formation of tubular structures is a unique feature of cowpea protoplasts, protoplasts of several plant species, both hosts and nonhosts to CPMV, were transfected with CPMV RNA and pMM48. Sixteen hours after transfection, these protoplasts were examined for the occurrence of viral RNA replication, protein expression, and the presence of tubules by immunofluorescence and electron microscopy. Whether viral RNA or pMM48 was used, tubular structures were found in protoplasts of all plant species tested (Table 1 and Fig. 4C and D). Remarkably, also in protoplasts from the cowpea line TVu 470, which is immune to CPMV infection but protoplasts of which do support virus replication (10), tubular structures are formed (Table 1). These results demonstrate that (i) CPMV RNA is capable of infecting protoplasts of a series of nonhost plants and (ii) the viral 48K protein has the property to induce tubules in these cells. Although large variations in the number of cells supporting RNA replication and the number of tubules among the different species were observed, no conclusions can be drawn from these differences, as no efforts were made to optimize the transfection efficiency for each protoplast system.

On the basis of the immunofluorescence staining of the tubules, it seems likely that the 48K protein is a structural component of the tubules. No evidence for the involvement of other (host) components has been found yet. Considering the variety of plant species in which tubules are formed, putative host components involved in this process are likely to be conserved proteins, e.g., cytoskeletal components.

The observation that tubular movement structures are induced in protoplasts from an immune cowpea and plant species (e.g., barley, cactus, and *A. thaliana*) that are not hosts for CPMV makes one wonder why CPMV is unable to systemically infect these plants. Possibly, the 58K protein, whose prime location in protoplasts appears to be the nucleus, has a function in the formation of tubular structures in the intact plant and/or in deregulation of cellular defense mechanisms. Thus, the 58K protein could be involved in modifying plasmodesmata prior to tubule formation by the 48K protein or in preventing occlusion of tubules by deposition of callose prior to virus release in the neighboring cell (14).

Whatever the function of the 58K protein might be, the results of this study unequivocally demonstrate that the 48K protein is the minimal requirement for tubule induction and formation. As such, this protein plays a key role in the cell-to-cell movement of CPMV and thus in systemic infection of plants. On the other hand, the ability of this gene product to induce tubules in protoplasts of a number of randomly chosen nonhost plants suggests that the 48K protein is not a major host range determinant, although an additional role of the 48K protein cannot be excluded.

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