NN08201, 2655

# STELLINGEN

1. In tegenstelling tot wat Ward *et al.*, 1997 beweren, zijn buizen geïnduceerd door het squash leaf curl virus niet uniek qua vorm en inhoud.
   Ward *et al.*, 1997. The geminivirus BII movement protein is associated with endoplasmic reticulum derived tubules in developping phloem cells. *Journal of Virology* **71**(5), 3726-3733.
   *Dit proefschrift.*

2. Voor plantenvirussen die transportbuizen induceren is afbraak van deze buizen een vereiste voor een succesvolle infectie van de plant.
   *Dit proefschrift.*

3. Het buizentransportsysteem heeft de toekomst!
   *De Gelderlander, 17 oktober 1998*
   *Algemeen Dagblad, 23 september 1998*

4. Anders dan Latvala *et al.* concluderen is het manteleiwit-gen van het nepovirus "blackcurrant reversion associated virus" niet 1598 nucleotiden lang.
   Latvala, S., Susi, P., Kalkinen, N. & Lehto, K. (1998). Characterization of the coat protein gene of mite-transmitted blackcurrant reversion associated nepovirus. *Virus research* **53**, 1-11.

5. Knoflook verlaagt de kans op (infectie)ziekten.
   Ankri, S. & Mirelman, D. (1999) Antimicrobial properties of allicin from garlic. *Microbes and Infection* **1** (2), 125-129.
   Koscielny, J., Klussendorf, D., Latza, R., Schmitt, R., Radtke, H., Siegel, G. & Kiesewetter, H. & G.R.P. Giessen Study (1999). The antiatherosclerotic effect of allium sativum. *Artherosclerosis* **144** (1), 237-249.
   Siegers, C.P., Steffen, B., Robke, A. & Pentz, R. (1999). The effects of garlic preparations against human tumor cell proliferation. *Phytomedicin* **6** (1), 7-11.

6. Een antroposofische leefwijze verlaagt de kans op allergieën.
   Alm (1999). Atopy in children of families with an antroposophic lifestyle. *Lancet* **353** (9163), 1485-1488.

7. Het zwart-wit gehalte van de kaft van een proefschrift zegt niets over het zwart-wit gehalte van de gedachten van de auteur van het proefschrift.

8. Zonder wegwerpluiers was dit proefschrift niet tot stand gekomen.

9. Mobiele telefonie redt levens en leidt tot toename van het aantal werkende moeders.

10. Het idee, dat zegeltjes sparen iets oplevert is een succesvolle meme.

11. De dood is belangrijker dan het leven.

Stellingen behorend bij het proefschrift: "Structure, morphogenesis and function of tubular structures induced by cowpea mosaic virus"
door Daniella Kasteel
10 september 1999,
Wageningen.

# STRUCTURE, MORPHOGENESIS AND FUNCTION OF TUBULAR STRUCTURES INDUCED BY COWPEA MOSAIC VIRUS

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NN08201, 2655

# STRUCTURE, MORPHOGENESIS AND FUNCTION OF TUBULAR STRUCTURES INDUCED BY COWPEA MOSAIC VIRUS

**Daniella T.J. Kasteel**

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Wageningen Universiteit,
Dr. C.M. Karssen,
in het openbaar te verdedigen op
vrijdag 10 september 1999
des namiddags om 4 uur in de aula.

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*samprapte samnihite kale,*
*nahi nahi raksati dukrnkarane.......*

*Wanneer het tijdstip van de dood gekomen is,*
*zal boekenkennis je niet beschermen.......*

Shankara (686-718 na Chr.)

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Chapter 1

# GENERAL INTRODUCTION

## INTRODUCTION

Plant viruses differ widely in particle morphology and genome organization and are classified accordingly in a considerable number of families and genera (Murphy *et al.* 1995). Despite these differences, plant viruses have in common that upon entry and replication in the initially infected cell they have to spread to surrounding cells to establish a systemic infection. As plant cells have rigid cell walls, and are only interconnected by the plasmodesmata, it is generally accepted that plant viruses specify "movement proteins" (MPs) that modify these plasmodesmata in such a way that they allow the passage of viral genomes or even mature virus particles (for reviews see Atabekov & Dorokhov, 1984, Hull, 1989, Atabekov & Taliansky, 1990, Maule, 1991, Deom *et al.*, 1992, Citovsky, 1993, Lucas and Gilbertson, 1994, Carrington *et al.*, 1996).

Cell-to-cell movement of plant viruses is an intriguing and complex process that has been increasingly analyzed over the past two decades. Thusfar, the main effort has been to identify viral factors involved in movement of viruses and different mechanisms have been postulated. In this chapter the mechanisms proposed for this particular event in the virus life cycle are presented, with emphasis on the so-called tubule-guided movement mechanism as this concerns the transport mechanism that is the central theme in this thesis.

## MECHANISMS OF VIRUS MOVEMENT

The distribution of virus through a plant requires two steps (Hull, 1989): movement over short distance from cell-to-cell and over long distance through the vascular system to different parts of the plant (for recent review see Seron & Haenni, 1996).

Different lines of evidence have led to the identification of specific viral factors that are involved in cell-to-cell movement of plant viruses. Amongst them are the so-called movement proteins (MPs) and the capsid proteins (CPs). For some plant viruses the necessity of a MP for cell-to-cell movement has been conclusively established by mutational analyses (e.g. cowpea mosaic virus (CPMV), Wellink & van Kammen, 1989; tobacco mosaic virus (TMV), Deom *et al.*, 1987; cauliflower mosaic virus (CaMV), Stratford & Covey, 1989, Thomas *et al.*, 1993, Perbal *et al.*, 1993). Their localization *in situ* has been investigated by fractionation studies and/or immunogold localization studies. The overall conclusion from these studies is that the MPs are most frequently present in the cell wall of infected plants, at or near plasmodesmata (e.g. CPMV, Wellink *et al.*, 1987, Van Lent *et al.*, 1990a; TMV, Tomenius *et al.*, 1987).

This supports the idea that virus movement occurs via these intercellular channels, which provide a cytoplasmic continuity between neighbouring cells and play a role in molecular trafficking (for reviews on plasmodesmata see Lucas & Gilbertson, 1994, Epel, 1994, Lucas, 1995, Lucas *et al.*, 1995, Zambryski, 1995, Overall & Blackman, 1996, Waigman *et al.*, 1997). However, plasmodesmata are too small for free passage of virions or viral genomes. This problem has already been pointed out in 1968 by Esau and in 1976 by Gibbs and is illustrated in Fig. 1. Even CPMV, one of the smallest plant viruses (28 nm in diameter), is too large to pass unmodified plasmodesmata, which have an effective diameter of approximately 3 nm (Lucas & Gilbertson, 1994). To allow passage of large molecules or structures, plasmodesmata have to undergo modification. Such modification is mediated by virus-encoded movement proteins (MPs).

Two modes of action of the MPs have been proposed in the past (Hull, 1989, Goldbach *et al.*, 1990); either the MP increases the gating capacity of the plasmodesmata without an apparent alteration of the plasmodesmata structure and facilitates the movement of viral nucleic acid, or the MP assembles into tubules which penetrate the plasmodesmata and can accommodate virus particles.

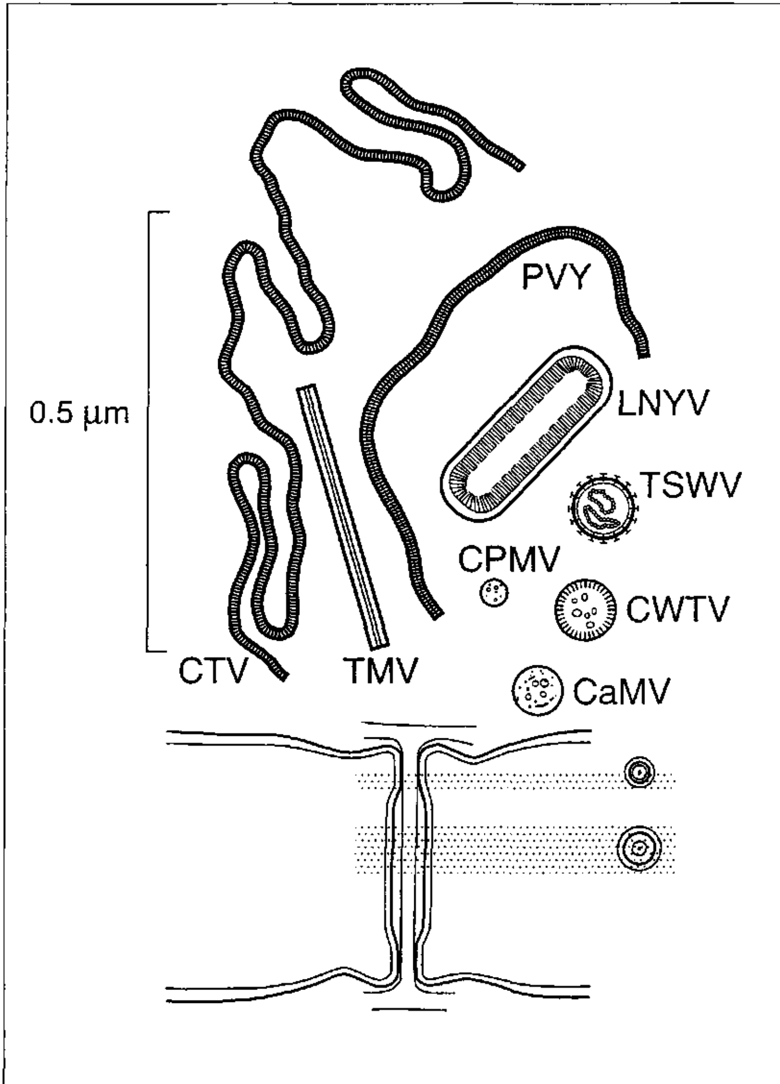


**Fig. 1.** The relative sizes of some plant viruses (top) compared to the size of a plasmodesma (bottom). CTV = citrus tristeza virus (2 µm x 10 nm), TMV = tobacco mosaic virus (300 nm x 18 nm), PVY = potato virus Y (750 nm x 11 nm), LNYV = lettuce necrotic yellows virus (220 nm x 80 nm), TSWV = tomato spotted wilt virus (80 nm), CWTV = clover wound tumor virus (70 nm), CaMV = cauliflower mosaic virus (50 nm), CPMV = cowpea mosaic virus (28 nm).

The first proposed mechanism (Citovsky & Zambryski, 1991, Deom *et al.*, 1992) is based on experimental evidence mainly obtained for TMV. Transgenic plants expressing the TMV-MP gene can complement movement-defective mutants (Deom *et al.*, 1987). As a result of the expression of the MP gene the gating capacity (size exclusion limit (SEL)) of plasmodesmata is increased (Wolf *et al.*, 1989). The TMV-MP binds nucleic acid *in vitro* (Citovsky *et al.*, 1990, 1992) and transports nucleic acid *in vivo,* upon microinjection, into adjacent cells (Waigman *et al.*, 1994). In addition, the MP of TMV has been localized in plasmodesmata, both in TMV-infected plants and in MP-transgenic plants (Tomenius *et al.*, 1987, Atkins *et al.*, 1991, Ding *et al.*, 1992, Moore *et al.*, 1992, Lapidot *et al.*, 1993). In the TMV model nucleic acids are moved from one cell to the other (Dorokhov *et al.*, 1984) via functionally modified plasmodesmata. Viruses that move according to this model do not require their capsid proteins for cell-cell transport (Dawson *et al.*, 1988, Takamatsu *et al.*, 1987).

The second model for virus movement ("tubule-guided cell-to-cell movement") is based on experimental evidence mainly obtained for CPMV. Electron microscopical observations of CPMV-infected plant material revealed a structural modification of plasmodesmata (Van der Scheer & Groenewegen, 1971, Kim & Fulton, 1975, Kim, 1979, Van Lent *et al.*, 1990a), in particular the assembly of a virion-containing tubule (35 nm in diameter, Van Lent *et al.*, 1990a), replacing the desmotubule normally present within the plasmodesmal channel (Robards, 1976). These tubules extend from one cell into the cytoplasm of the neighbouring cell (see Fig. 2) and can be labeled with antiserum which reacts with the RNA-2 encoded, in sequence largely overlapping 48 kDa and 58 kDa proteins of CPMV (Van Lent *et al.*, 1990a, for translation strategy of CPMV and additional data see Fig. 3 and also Goldbach & Wellink, 1996). The particular antiserum used in these studies however, could not discriminate between the 48 kDa and the 58 kDa proteins. At the onset of this PhD research it was therefore not clear whether both 58 kDa and 48 kDa proteins, or only one of them was present in the tubules and represented the CPMV MP. Evidence for a different location of the 48 kDa and 58 kDa protein has been presented (Wellink *et al.*, 1987; Holness *et al.*, 1989; Rezelman *et al.*, 1989). The 48 kDa protein was present in the cytoplasmic and membrane fraction of infected protoplasts, whereas the 58 kDa protein was only found in the cytoplasmic fractions (Rezelman *et al.*, 1989). Furthermore, the 48 kDa protein is the only non-structural viral protein detected in the culture medium of infected protoplasts (Wellink *et al.*, 1987). These data suggest that only the 48 kDa is present in tubules and not the 58 kDa protein.

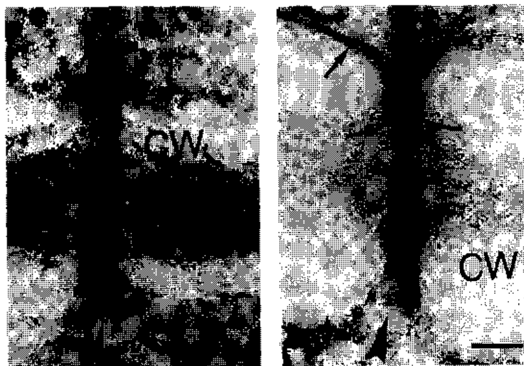


**Fig. 2.** Tubular structures containing virus particles in CPMV infected tissue. Bar represents 100 nm. CW = cell wall. Small arrows indicate the plasmamembrane. Large arrow indicates virusparticles accumulating near one end of the tubule.

## COWPEA MOSAIC VIRUS

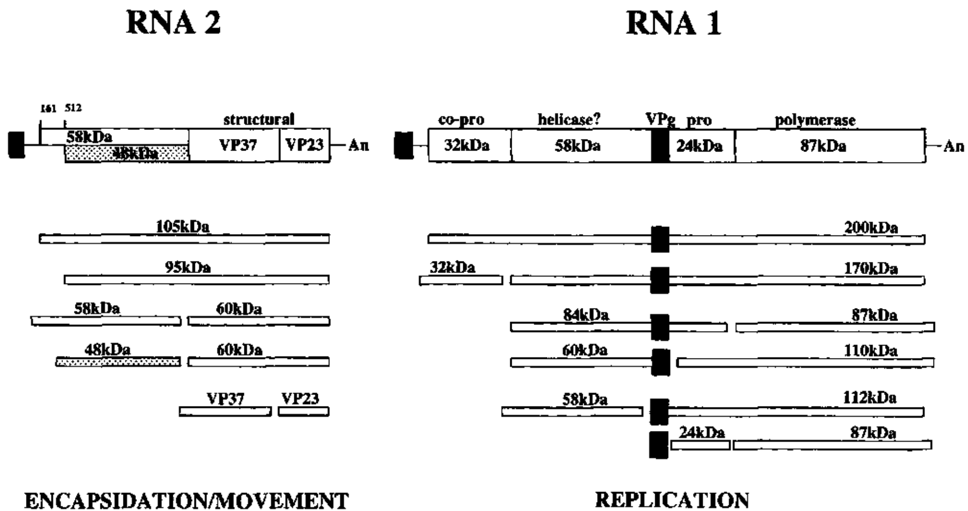


**Fig. 3.** Genomic organization of the bipartite genome of CPMV. Cowpea mosaic virus (CPMV) is the type member of the genus Comovirus (Murphy *et al.*, 1995). Comoviruses have small icosahedral particles with a diameter of 28 nm. Three types of particles (B, M and T component) exist that have an identical protein coat (60 copies of each of the two different capsid proteins, Wu & Bruening, 1971), but differ in RNA contents. B and M component each contain a segment of the single stranded bipartite RNA genome (RNA 1 and 2 respectively). T component consists of empty protein shells. Both B and M component, or their RNA species, are necessary for infection of plants (Van Kammen, 1968, De Jager, 1976). The genomic RNAs have a small protein (VPg, indicated by black boxes) covalently linked to their 5'ends and a poly-A tail at their 3' ends. CPMV RNAs are translated into polyproteins which are subsequently cleaved into the functional proteins by virus-encoded protease (Goldbach & Van Kammen, 1985, Vos *et al.*, 1988). RNA 1 encodes all the functions necessary for replication of the virus (Goldbach *et al.*, 1980, Eggen & Van Kammen, 1988) and can replicate independently of the RNA 2 in protoplasts. RNA 2 is essential for successful infection of plants (Rezelman *et al.*, 1982) and encodes all the proteins involved in transport and encapsidation of the virus. It encodes a 48 kDa protein (MP), an overlapping 58 kDa protein and the two capsid proteins, VP23 and VP37 (Franssen *et al.*, 1982) via two overlapping polyproteins of 95 and 105 kDa. All RNA 2-encoded proteins are involved in the cell-to-cell movement of the virus (Wellink & van Kammen, 1989). Whenever these proteins are mutated there is no systemic infection of the plant. The infection is then restricted to the initially infected cell.

A major breakthrough in CPMV movement research was the finding that movement tubules are induced in isolated plant protoplasts upon infection with CPMV (Van Lent *et al.*, 1991). Such tubules were visualized by immunofluorescence microscopy, using the antiserum against the overlapping 48 kDa/58 kDa proteins and they appear to extend from the surface of the protoplasts into the culture medium (Fig. 4). In addition, staining of the nucleus was observed (van Lent *et al.*, 1991). The tubules induced in protoplasts, appear to be morphologically identical to tubules observed in infected plant tissue. They are approximately 35 nm in diameter, contain a single row of virus particles and are enveloped by the plasma membrane.

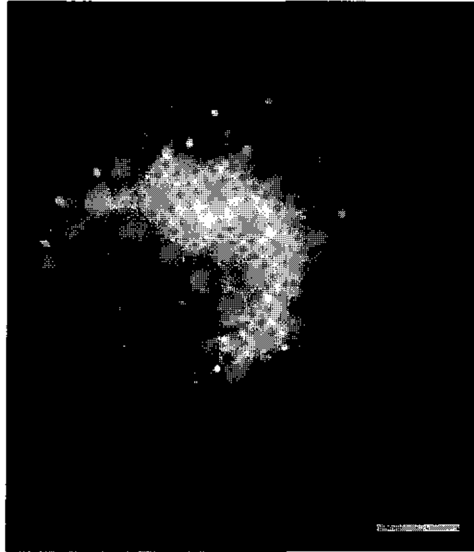

**Fig. 4.** Immunofluorescent image of a CPMV infected protoplast, labeled with anti-48 kDa/58 kDa serum. Many tubular structures extend from the surface of the protoplast. Bar represents 10 μm.

Thus, in the CPMV model (see also Fig. 5) the virus is proposed to move from cell-to-cell as mature virions, implying that in addition to the MP also CPs are essential for cell-to-cell movement (Wellink & van Kammen, 1989).

Recent studies on intercellular movement of other plant viruses suggest that the two above described mechanisms for cell-to-cell movement no longer sufficiently explain the movement of all plant viruses. Several plant viruses seem to employ a mechanism of movement which exhibits properties of both mechanisms described above. CaMV for example forms MP-containing tubules with virions (Conti *et al.*, 1972, Linstead *et al.*, 1988) like CPMV, but its MP also has *in vitro* nucleic acid binding capacity like the TMV MP (Citovsky & Zambryski, 1991). For potato virus X (PVX) the MP increases the SEL of plasmodesmata (Angell *et al.*, 1996), a characteristic attributed to TMV-like MPs, but its CP is necessary for cell-cell movement and located in plasmodesmata, as found for CPMV. From these examples it is clear, that more research on cell-to-cell movement of plant viruses is needed to further define the different mechanisms. In this thesis, the tubule-guided movement mechanism of CPMV is studied in more detail.

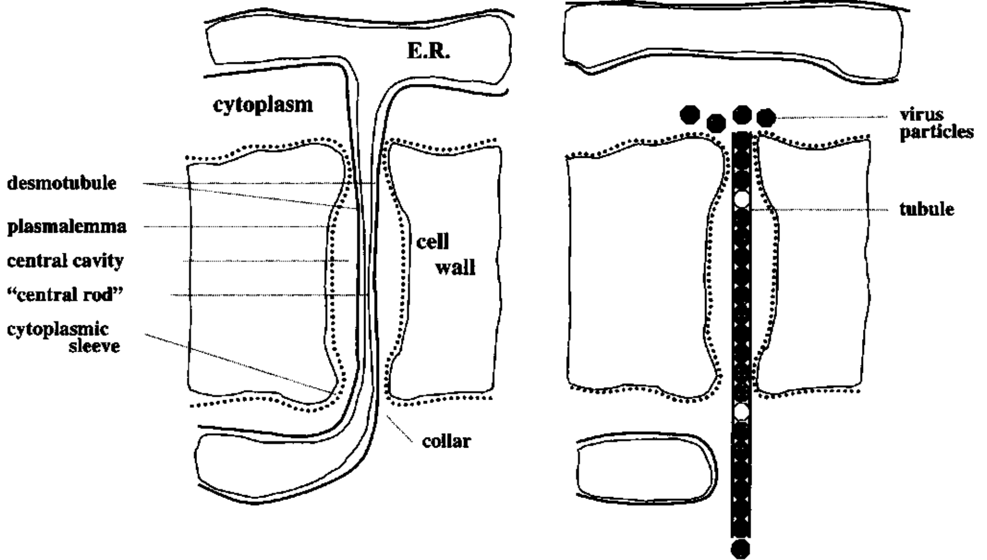


**Fig. 5.** Schematic representation of a normal (left) and a modified plasmodesma (right). The CPMV induced modification consists of removal of the desmotubule and the formation instead, of a tubule containing virus particles.

## OUTLINE OF THESIS

From the previous paragraphs it has become clear that at the onset of the PhD study presented in this thesis it was proposed that during systemic infection of host plants comovirus CPMV moves from cell-to-cell as mature particles through tubules which assemble in plasmodesmata. However, it was not yet known whether the 48 kDa protein or the 58 kDa protein, or both, have a function in this process. This, due to the complication that their coding sequences in RNA 2 overlap, which complicates serological distinction. Therefore, as a start, a deletion analysis on the coding sequence of these overlapping proteins was performed, using a full-length "infectious" cDNA clone of RNA 2 (Chapter 2). This analysis supported the view that the 48 kDa protein represents the MP of CPMV, although a role of the 58 kDa protein in intercellular transport could not be completely ruled out. In Chapter 3 the role of the 58 kDa protein in the virus infection cycle was analyzed. Further evidence was obtained that this protein is not involved in viral movement, but no conclusive proof could again be obtained. Therefore full attention was given to investigations which would demonstrate that only the 48 kDa protein is essential for cell-to-cell movement. This was done using two different approaches. Firstly, the 48 kDa gene was heterologously expressed using the baculovirus/insect cell system (Chapter 4). Secondly, the transport tubules were quantitatively isolated and their protein composition analyzed (Chapter 5). For this latter approach infected cowpea protoplast batches were used, as previous research from our group had shown that on the surface of these single cells large quantities of transport tubules are assembled. The results obtained in both approaches demonstrate that only the 48 kDa protein is involved in the tubule-guided, intercellular movement of CPMV, and thus represents the comoviral MP, whereas a structural role of any host factor could be excluded. In Chapter 6 the formation of similar tubular structures by two other viruses, brome mosaic virus (BMV) and alfalfa mosaic virus (AMV) is described. These two viruses are genetically more closely related to TMV (a virus which does not move from cell to cell by a tubule-guided mechanism) than to CPMV. As both BMV and AMV form virus-containing tubular structures in protoplasts it may be hypothesized that aggregation of MP into tubules is a more common feature of plant virus MPs than previously thought. This hypothesis is further supported by evidence from other viral models and by additional experimental data, as discussed in Chapter 7.

Chapter 2

# THE INVOLVEMENT OF COWPEA MOSAIC VIRUS RNA-2-ENCODED PROTEINS IN TUBULE FORMATION

This chapter has been published in a slightly condensed form as: Daniella Kasteel, Joan Wellink, Jan Verver, Jan van Lent, Rob Goldbach and Ab van Kammen (1993). The involvement of cowpea mosaic virus M RNA-encoded proteins in tubule formation. *Journal of General Virology* **74**, 1721-1724.

## SUMMARY

On the surface of cowpea protoplasts inoculated with cowpea mosaic virus (CPMV), tubular structures containing virus particles have been found. Such tubular structures are thought to be involved in cell-to-cell movement of CPMV in cowpea plants. To study the involvement of the 58 kDa/48 kDa and capsid proteins of CPMV in the formation of tubular structures, mutations were introduced into cDNA clones of RNA-2 from which infectious transcripts could be derived. No tubules were found on protoplasts inoculated with a mutant that fails to produce the 48 kDa protein nor with a mutant that has a deletion in the 48 kDa coding region, suggesting that the 48 kDa protein is essential for this process. However, a possible role of the 58 kDa protein in tubule formation could not be excluded. A mutant that fails to produce the capsid proteins did produce tubules and therefore the capsid proteins are not involved in the formation of the tubular structures. Electron microscopic analysis revealed that the tubules produced by this mutant are, apart from the absence of virus particles, morphologically identical to the tubules formed by the wildtype virus.

## INTRODUCTION

Cowpea mosaic virus (CPMV) has a bipartite plus-strand RNA genome (Eggen & van Kammen, 1988). The larger RNA-1 encodes the functions necessary for the replication of the RNAs and is able to replicate independently of the RNA-2 in protoplasts (Goldbach *et al.*, 1980). The RNA-2 is essential for successful infection of plants and encodes proteins that are involved in cell-to-cell movement of the virus (Rezelman *et al.*, 1982; Wellink & van Kammen, 1989). Both RNAs are translated into polyproteins that are cleaved into functional proteins by a RNA-1-encoded protease (Vos *et al.*, 1988). The RNA-2 is translated into two polyproteins, of $M_r$s 105 kDa and 95 kDa, that are processed to give the overlapping 58 kDa and 48 kDa proteins and the 60 kDa precursor of the capsid proteins VP37 and VP23 (Franssen *et al.*, 1982). Using infectious transcripts derived from cDNA clones of the RNA-2 it was shown that the 58 kDa/48 kDa protein pair and the capsid proteins are required for cell-to-cell movement of CPMV (Wellink & van Kammen, 1989).

In CPMV-infected plants tubular structures containing virus particles are observed in the plasmodesmata (van Lent *et al.*, 1990*a*, 1991). These tubular structures can be labeled with antiserum against the 58 kDa/48 kDa proteins and are thought to play a major role in cell-to-cell movement of the virus. Recently, van Lent *et al.* (1991) showed that these tubular structures are not only induced in infected cowpea plant cells but also in cowpea protoplasts inoculated with CPMV. In these protoplasts the tubules are formed at the surface of the cell and protrude into the culture medium. Since the 48 kDa protein is detected in the medium of protoplasts inoculated with CPMV (Wellink *et al.*, 1987), it is plausible that the 48 kDa movement protein is a structural component of the tubular structures. Whether the tubular structures also contain other viral proteins or host proteins has not yet been established.

In this paper we describe the use of RNA-2 mutants of CPMV to study the involvement of the 58 kDa/48 kDa proteins and the capsid proteins of CPMV in the formation of tubular structures on protoplasts.

## METHODS

### *Construction and description of RNA-2 mutants*

All DNA manipulations were essentially as described by Sambrook *et al.* (1989). Plasmids pTM1G and pTB1G contain full-length cDNA copies of the RNA-2 and RNA-1 of CPMV respectively, from which infectious RNA can be obtained by transcription with T7 RNA polymerase (Eggen *et al.*, 1989). Plasmid pBS'M (O. Le Gall, unpublished) contains a full-length cDNA clone of the RNA-2 derived from pTM1G in pBSKS$^+$ (Stratagene). Previous nomenclature was B RNA and M RNA for RNA-1 and RNA-2 respectively. At the time of publication of this chapter the nomenclature for RNA 2 was still M RNA, hence RNA mutants in this chapter are indicated with "M".

In order to obtain a mutant RNA-2 which coded only for the 58 kDa/48 kDa proteins, pTM58S was constructed by site-directed mutagenesis of pBS'M with the oligonucleotide 5' GGC-AAA-CAA-GTT-**TTA-GGC-CTA**-TTG-TGG-AAA-AGC 3', as described by Kunkel (1985). This resulted in the creation of two stop codons (shown in bold) downstream of the 58 kDa/48 kDa coding sequence and a *Stu*I recognition site (underlined) (Fig. 1). Starting from the plasmid pTM58S a fragment was removed from the newly created *Stu*I site up to *Nae*I (3172) resulting in pTM58SΔ5 (Fig. 1).

The two other RNA 2 mutants used in this study contain mutations in the 58 kDa/48 kDa coding region. MΔAUG2/3 lacks the two start codons used for initiation of translation of the 95 kDa (48 kDa) protein (construction of this mutant, in which the AUG codons at positions 512 and 524 have been changed into UUC and AGU respectively, will be described in detail elsewhere) and MΔP RNA has a deletion of 486 nt in the coding region for the 58 kDa/48 kDa proteins (Fig. 1; Wellink & van Kammen, 1989). The integrity of the RNA-2 mutants was tested by *in vitro* translation in rabbit reticulocyte lysates. M58S and M58SΔ5 RNA produced 58 kDa and 48 kDa proteins, MΔAUG2/3 RNA produced a 105 kDa protein and MΔP RNA produced 88 kDa and 78 kDa proteins as expected (data not shown).

### *Testing of RNA-2 mutants in protoplasts and plants*

The infectivity and tubule forming capacity of the RNA-2 mutants was tested by inoculation of cowpea protoplasts with the transcripts together with the wild-type RNA-1 derived from pTB1G as described by Eggen *et al.* (1989). Infection of the protoplasts was established by an immunofluorescence test as described by van Lent *et al.* (1991) using anti-24 kDa serum to check for the replication of RNA-1 and anti-58 kDa/48 kDa and anti-CPMV sera to detect replication of RNA-2. Tubule formation was followed by immunofluorescence microscopy using anti-58 kDa/48 kDa serum and by electron microscopy (van Lent *et al.*, 1991). This anti-58 kDa/48 kDa serum was raised against a synthetic peptide of the 30 C-terminal residues of both the 48 kDa and 58 kDa proteins (Wellink *et al.*, 1987) and is able to detect both the 48 kDa and 58 kDa protein species simultaneously. However, in immunogold electron microscopy its reaction with tubular structures is limited to broken and disintegrated tubules (van Lent *et al.*, 1991).

The replication level of mutants M58S and M58SΔ5 was tested by inoculation of protoplasts with transcripts derived from the plasmids followed by Northern blot analysis of RNA extracted from the protoplasts at 70h post-infection (Wellink & van Kammen, 1989).
The ability of M58SΔ5 RNA to support an infection of a whole plant was tested by inoculation of primary leaves of 10-day-old cowpeas with RNA-1 and this mutant RNA-2, followed by Western blot analysis (see Wellink & van Kammen, 1989).

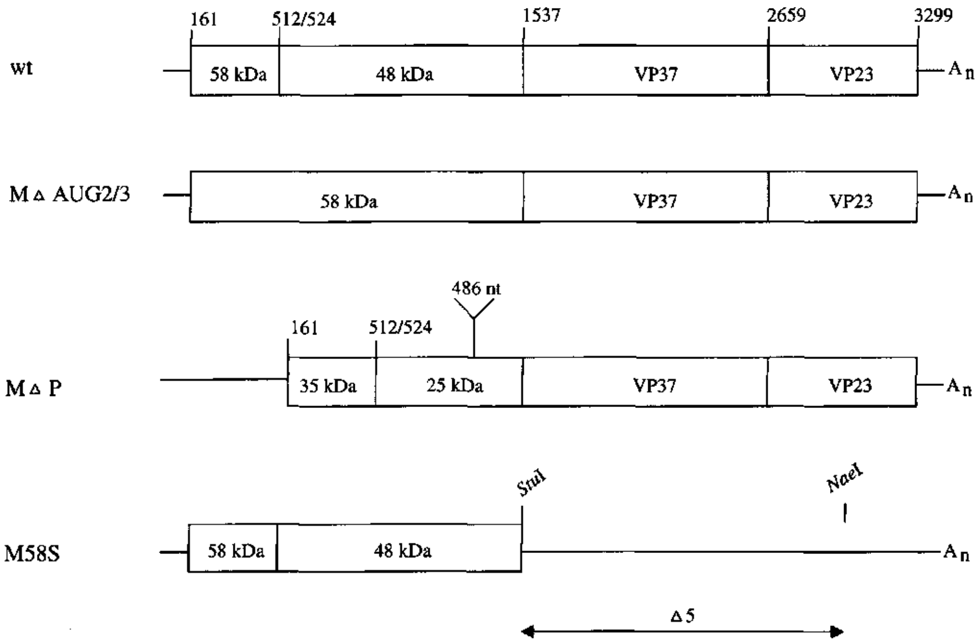


**Fig. 1.** Schematic representation of RNA-2 mutants used. Open reading frames are indicated as open bars. The start codons at positions 512 and 524 are modified in MΔAUG2/3 and therefore this mutant produces only the 58 kDa protein and the capsid proteins. MΔP contains a deletion in the 58 kDa/48 kDa coding region and produces truncated 48 kDa and 58 kDa proteins. M58S contains two stopcodons downstream of the 58 kDa/48 kDa coding region and fails to produce the capsid proteins. Starting from this mutant the mutant M58SΔ5 was constructed, which contains a deletion in the sequence coding for the capsid proteins. The restriction sites used for the construction of this mutant are indicated. wt, Wild-type.

## RESULTS AND DISCUSSION

In order to obtain a mutant RNA-2 which coded only for the 58 kDa/48 kDa proteins, pTM58S was constructed. The infectivity of this RNA-2 mutant was concluded to be very low because no fluorescent protoplasts could be found with anti-58 kDa/48 kDa serum. We reasoned that the remaining long non-coding sequence downstream of the 58 kDa/48 kDa coding region might cause instability of the transcripts derived from pTM58S and therefore decided to construct a plasmid (pTM58SΔ5) with a deletion in this sequence.

The results showed that the replication level increased after the deletion in the capsid protein precursor coding region (data not shown). When judged by immunofluorescence, the percentage of cells infected with M58SΔ5 RNA was very similar to the percentage obtained with wild-type RNA-2, which was arbitrarily set at 100% (Table 1). Therefore M58SΔ5 RNA was used in further experiments. The infectivity of MΔP RNA and MΔAUG2/3 RNA was about 75% and 50% respectively, as determined by immunofluorescent staining (Table 1).

To test whether the M58SΔ5 RNA was still able to support infection of a whole plant, leaves were inoculated with this mutant. As expected, the mutant was not able to spread from cell to cell as viral proteins could not be detected in the inoculated leaves.

To study the involvement of the capsid proteins and the 58 kDa/48 kDa proteins in the formation of the tubular structures on protoplasts, protoplasts were inoculated with the RNA-2 mutants together with RNA-1 and screened for the presence of tubular structures by immunofluorescence microscopy with the anti-58 kDa/48 kDa serum and electron microscopy, as described by van Lent *et al.* (1991). Using the immunofluorescence test with anti-58 kDa/48 kDa serum, no tubular structures were found on protoplasts inoculated with the 58 kDa/48 kDa protein mutants MΔP and MΔAUG2/3 or RNA-1 alone, whereas the control protoplasts inoculated with wildtype RNA-2 and the protoplasts inoculated with the capsid protein mutant M58SΔ5 did show tubular structures (Table 1). The absence of tubular structures on protoplasts inoculated with the mutants MΔP and MΔAUG2/3 and RNA-1 alone was also confirmed by electron microscopy. These results suggest that the 48 kDa protein is involved in tubule formation. However, it is possible that the 58 kDa protein also has a role in this process.

The 58 kDa protein produced by MΔAUG2/3 RNA is modified at three positions due to mutagenesis of the start codons of the 48 kDa protein (Met512 to Phe, Met524 to Ser and Ser525 to Thr) which could have resulted in a protein that was no longer able to induce tubular structures. Mutations in the N terminus of the 58 kDa protein abolish the infectivity of the RNA (Wellink & van Kammen, 1989; Holness *et al.*, 1989; van Bokhoven *et al.*, 1993) and therefore it is not possible, using mutants, to study whether the expression of only the 48 kDa protein results in tubule formation.

**Table 1.** *Analysis of protoplasts inoculated with RNA-2 mutants of CPMV by immunofluorescence and electron microscopy*

| RNA | Protoplasts stained with anti-CPMV serum (%) | Protoplasts stained with anti-58 kDa/48 kDa serum (%) | Tubular structures♣ | Staining of the nucleus♣♣ |
|---|---|---|---|---|
| RNA-2 + 1 | 100* | 100 | + | + |
| RNA-1 | 0 | 0 | - | - |
| M58SΔ5 + RNA-1 | 0 | 100 | + | + |
| MΔP + RNA-1 | 75 | 75 | - | ?♦ |
| MΔAUG2/3 + RNA-1 | 50 | 50 | - | + |

* The proportion of protoplasts inoculated with wild-type RNA-2 and RNA-1 that stained with anti-CPMV serum and anti-58 kDa/48 kDa serum was arbitrarily set to 100% and corresponds to 35 % of the protoplasts.

♣ As determined by immunofluorescence and electron microscopy.

♣♣ With the anti-58 kDa/48 kDa serum.

♦ Protoplasts inoculated with this mutant showed staining throughout the cytoplasm and occasional staining of the nucleus

Previously, it has been proposed that the 48 kDa protein of CPMV is a structural component of the tubule (van Lent *et al.*, 1991). Aggregation of 48 kDa proteins that are produced in large quantity during the infection process could be the mechanism that leads to the generation of the tubular structures. In addition, the 48 kDa protein may induce a host protein to copolymerize (a cytoskeleton protein for example).

As expected, electron microscopic examination of tubular structures induced by M58SΔ5 transcripts revealed that these structures did not contain virus particles due to the absence of capsid proteins (Fig. 2*c*,*d*). Besides this they appeared to be morphologically identical (dimensions of the tubule wall, presence of the plasma membrane) to the tubular structures present on protoplasts inoculated with wild-type RNA-2 and RNA-1 (Fig. 2). The capsid proteins therefore have no role in the induction and growth of the tubules. On the other hand it is very likely that a specific interaction between the virus particles and the tubule wall takes place because the particles always appear to be neatly arranged in the tubules (Fig. 2*a*,*b*; van Lent *et al.*, 1990*a*, 1991). An intriguing question which remains to be answered is whether virus particles actually move through the tubules.

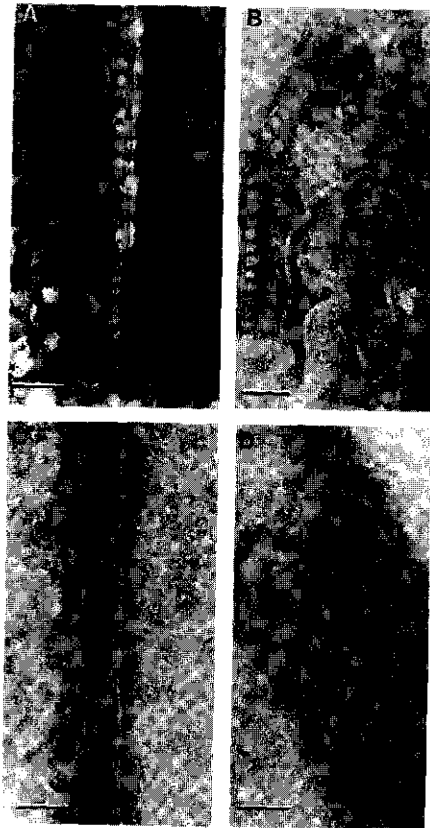


**Fig. 2.** Electron micrographs of negatively stained virus particle-containing tubular structures (surrounded by the plasma membrane) from protoplasts inoculated with wild-type CPMV RNA (*a*) or RNA-2 and RNA-1 transcripts (*b*). Empty tubular structures of protoplasts inoculated with M58SΔ5 and RNA-1 transcripts (*c* and *d*). Bar markers represent 0.1 μm.

Previously, staining with the anti-58 kDa/48 kDa serum of the nucleus of protoplasts inoculated with wild-type CPMV has been observed (van Lent *et al.*, 1991). Also, in protoplasts inoculated with the mutants MΔAUG 2/3 and M58SΔ5, staining of the nucleus could be observed which in the case of MΔAUG 2/3 was somewhat weaker than for the wild-type (Table 1). Protoplasts infected with MΔP RNA showed staining with the anti-58 kDa/48 kDa serum over the entire protoplast, probably because the 58 kDa and 48 kDa proteins produced by this mutant are not intact and accumulate in the cytoplasm. Occasionally, fluorescent staining of the nucleus could be observed as well. The results strongly suggest that the fluorescent staining of the nucleus is mainly due to the 58 kDa protein. Whether the presence of the 58 kDa protein in the nucleus of cowpea protoplasts is essential for the function of this protein or is an artefact of the protoplast system is not clear.

Chapter 3

# THE 58 kDa PROTEIN OF COWPEA MOSAIC VIRUS IS NOT INVOLVED IN TUBULE FORMATION

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## SUMMARY

The role of the RNA-2 encoded 58 kDa protein of cowpea mosaic virus (CPMV) in the infection process has not yet been elucidated. Former studies showed that the N-terminal domain of this protein is required for replication of RNA 2 and that this protein may accumulate in the nucleus of the infected cells. To further investigate the role of the 58 kDa protein in the viral infection cycle, it was aimed to obtain a specific antiserum that could discriminate between the 58 kDa and 48 kDa proteins, despite their overlapping sequences. As an alternative approach to study its localization, the 58 kDa protein was produced from a transient expression vector, to follow its intracellular targeting without the background of a full infection process. The results obtained fit the view that the 58 kDa protein has no apparent function in viral cell-to-cell movement.

## INTRODUCTION

Mutational analysis (Kasteel *et al.*, 1993, Chapter 2) showed that the 48 kDa protein encoded by RNA-2 of CPMV is essential for tubule formation at the surface of infected protoplasts. The capsid proteins, and therefore virions, have no role in this process. Furthermore, evidence was obtained that the RNA-2 encoded 58 kDa protein is not able to induce tubule formation, although this protein contains the entire 48 kDa sequence (see Chapter 1; Fig. 3 for translation strategy of CPMV). The exclusive function of the 48 kDa protein as tubule forming protein was confirmed by transient expression of the 48 kDa gene in protoplasts (Wellink *et al.*, 1993), leading to formation of tubules indistinguishable from those formed during the viral infection process, except that no virions are present inside.

The results obtained sofar suggest that the 48 kDa protein represents the single, viral movement protein (MP), generating a plasmodesma penetrating tubule for transport of virions from cell-to-cell. The function of the 58 kDa protein has remained unclear, although van Bokhoven *et al.* (1993) showed that the N-terminal domain of the protein is essential for replication of RNA 2.

Our results (Chapter 2 and Wellink *et al.*, 1993) show that the 58 kDa protein is mainly located in the nucleus of infected cells, a location which seems not to correspond with a function in the viral replication cycle, as this process takes place in the cytoplasm.

Here, we report further studies to characterize the intracellular behaviour of the 58 kDa protein as to get clues about its possible function(s) during the viral infection process. To this end a new antiserum against the entire 58 kDa protein was produced, and different approaches were tested, using both *E.coli* expressed protein sequences and synthetic peptides, to produce antibodies which would selectively detect the 58 kDa but not the 48 kDa protein.

## METHODS

***Expression of CPMV 58 kDa protein and its 10 kDa N-terminus in E.coli***

To produce the 58 kDa protein and its 10 kDa N-terminus in *E.coli*, plasmid pET-58K and pET-10K were constructed, respectively. The fragment encoding the 58 kDa protein was synthesized by PCR from plasmid pTM58S (Chapter 2, Kasteel *et al.*, 1993) using the primers 5' GGCACGTGATCACCATGGCTTCTTTCACTGAAGC 3' and 3' CGAAAA GGTGTTATCCGGACTAGTGATCCG 5'. The PCR fragments were digested with *Nco*I and *Bcl*I and cloned into a pET11t vector (Novagen), digested with *Nco*I and *BamH*I.

The fragment encoding the N-terminal 10 kDa part of the 58 kDa protein was synthesized by PCR amplification from mutant pTM58S (Chapter 2, Kasteel *et al.*, 1993) using the primers 5' GGCACGTGATCACCATGGCTTCTTTCACTGAAGC 3' and 3'GACGGGTTTAAA CTTATCCGGACTAGTGATCCG 5'. The PCR fragments were digested with *Nco*I and *Bcl*I and subsequently cloned into the vector pET11t (Novagen), digested with *Nco*I and *BamH*I.

Plasmids pET-58K and pET-10K were obtained in *E.coli* strain DH5α. For synthesis of protein, *E.coli* strain BL21 was transformed with either plasmid pET-58K or pET-10K. The synthesis of protein was induced by adding IPTG (1 mM) to 100 ml culture of *E.coli.* SDS-PAGE analysis and Western blot analysis (using the new 48 kDa/58 kDa antiserum) was performed to monitor the production of protein.

For the purification of the 58 kDa protein, the pellet containing the IPTG-induced *E.coli* was resuspended in 3 ml of lysis buffer (5% SDS, 50mM Tris pH 7.5) and 3 ml of 2 times concentrated sample buffer (20mM Tris, 2 mM EDTA, 4% SDS, 20% glycerol, 0.0002 % bromophenol blue, pH 6.8). After 15 min. boiling, 600 µl ß-mercapto-ethanol was added and the sample was loaded on a 8% acrylamide gel in a Prep Cell apparatus (Biorad, model 491, 37 mm ID column, 50 ml monomer, length of gel 6 cm). Electrophoresis was performed in 8 h at 50 mA. After elution of the blue marker dye (5.5 h after starting the electrophoresis), 1 ml fractions were collected at a rate of 0.5 ml per min. These fractions (7.5 ml) were analyzed on 8% SDS-polyacrylamide gels. Proteins were visualized either by staining with Coomassie brilliant blue (Fig. 1a) or by Western blot analysis (Fig. 1b) using an antiserum against the 30 C-terminal residues of the 48 kDa/58 kDa proteins (Wellink *et al.*, 1987). Fractions containing the purified 58 kDa protein (fractions 16, 17 and 18, Fig. 1) were freeze-dried and resuspended in 120 µl water. The sample was then desalted using Millipore Ultrafree-MC centrifugal-driven filters. The final protein concentration was determined with a Bio-Rad protein assay. Two independent purifications gave a total of 230 µg 58 kDa protein.

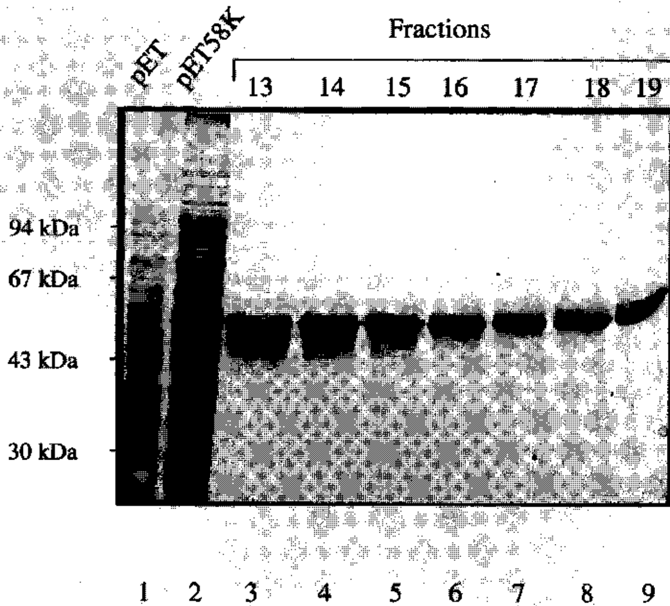
***Production and testing of antisera against the 58 kDa protein***

For the production of an antiserum against the entire 58 kDa protein a rabbit was injected hyperdermically with 80 µg of the *E.coli*-expressed, purified 58 kDa protein mixed with Freund (Brunschwig) adjuvant, followed, three weeks later, by injection of another 150 µg protein. Bleeding of the rabbit took place after two weeks. The specificity of the antiserum was tested on Western blots of samples of purified 58 kDa protein. Controls consisted of *E.coli* cells transfected with the empty pET11t vector.

Although a previous attempt to produce a 58 kDa specific antiserum, using a peptide corresponding to the N-terminus of the 58 kDa protein, failed, a new attempt was made using a similar approach. Two peptides corresponding to regions in the 10 kDa N-terminal part unique to the 58 kDa protein were made (Severn Biotech. Ltd). Both peptides were dissolved in water and 1 mg of each peptide was mixed with Freund adjuvant and injected hyperdermically into rabbits. After two weeks this procedure was repeated. Bleeding of the rabbits took place after two weeks. The antisera obtained were tested on spotblots, containing 10 µl (1 mg/ml) of the synthetic peptides.

The new antisera, raised against the entire 58 kDa protein and against the peptides, were all tested by immunofluorescence microscopy on CPMV-infected cowpea (*Vigna unguiculata*) protoplasts (van Lent *et al.*, 1991) and by immunogold electron microscopy on ultrathin sections of CPMV-infected cowpea leaves (van Lent *et al.*, 1990a). Controls consisted of mock-inoculated protoplasts and healthy leaf material respectively.

a)




b)

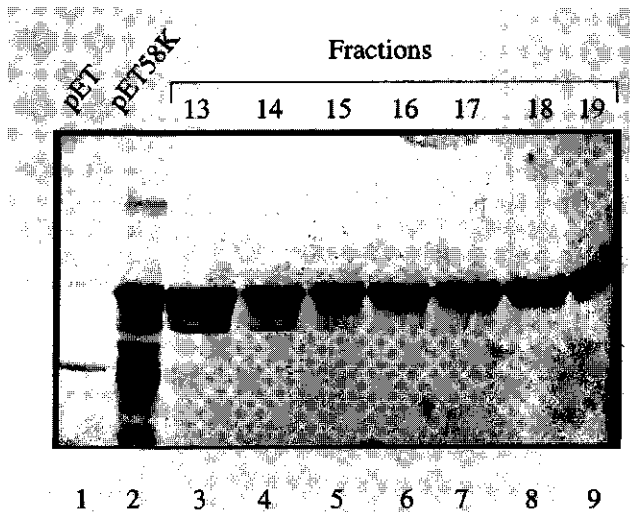



**Fig. 1.** SDS-polyacrylamide gel stained with Coomassie brilliant blue *(a)* and Western blot analysis using the anti-48 kDa/58 kDa serum *(b)* of the fractions 13 to 19 (lane 3 to 9) of the Prep Cell purification of the 58 kDa protein. Lane 1 (in *a* and *b*) contains proteins of *E.coli* transfected with pET 11t vector without insert, lane 2 (in *a* and *b*) proteins of *E.coli* transfected with pET-58K.

***Construction and testing of vector pMon58***

For transient expression in cowpea protoplasts the 58 kDa coding sequence was amplified by PCR from mutant MΔAUG2/3 (Chapter 2, Kasteel *et al.*, 1993) using the primers 5'GGCACGTGATCACCATGGCTTCTTTCACTGAAGC 3' and 3'CGAAAAGG TGTTATCCGGACTAGTGATCCG 5'. The PCR fragments were digested with *BclI* and cloned into the vector pMon999 (a gift from C. Hemenway, Monsanto Company), digested with *BamHI*. Plasmid pMon58 was obtained in DH5α. The integrity of the plasmid was verified by sequence analysis.

Expression was studied by transfecting samples of cowpea protoplasts ($10^6$ cells) with 10 μg of plasmid pMon58, 10 μg of pMon999 or with water. The isolation and transfection of protoplasts were essentially as described by Wellink *et al.* (1993). At 24 h p.i. transfected protoplasts were analyzed by immunofluorescence microscopy essentially as described by van Lent *et al.* (1991) using the new anti-48 kDa/58 kDa serum described above.

## RESULTS

***Testing of a new antiserum raised against E.coli expressed 58 kDa protein***

A new antiserum against the overlapping 48 kDa/58 kDa proteins was produced to overcome the limitations of the existing anti-48 kDa/58 kDa serum raised against the 30 C-terminal residues of both proteins (see Chapter 2). This latter antiserum reacted only with tubular structures which were broken or disintegrated (van Lent *et al.*, 1991), and gave an unexpected positive signal with nuclei of infected protoplasts using immunofluorescence, but not in immunogold labeling studies. In view of these, on first sight unexplainable results, a new antiserum was produced but now raised against the complete, *E.coli* expressed, 58 kDa protein sequence (see Fig. 1). After verifying its specificity by Westernblot analysis on purified 58 kDa protein (result not shown) this new antiserum was tested on CPMV infected protoplasts, using different dilutions. To allow a comparison, protoplasts were also treated with the pre-existing antiserum raised against a peptide consisting of the 30 C-terminal aminoacids of the 58 kDa/48 kDa proteins (Chapter 2).

**Fig. 2.** Immunofluorescent images of CPMV infected protoplasts 48h after infection treated with the new antiserum against the 58 kDa protein produced in *E.coli*, revealing tubular structures (*a*) and the nucleus (*b*). (*c*) Electron micrograph of a tubular structure immunogold labeled with the same antiserum. Bars represent 5 μm (*a,b*) and 100 nm (*c*).

Infected protoplasts treated with the new antiserum showed a clear staining of tubular structures and nucleus (Fig. 2*a,b*), whereas mock-inoculated protoplasts did not show any staining at all. Infected protoplasts treated with the pre-existing antiserum showed the same pattern of fluorescence, although less bright (as shown in Fig. 3*a,b*). Note that the two different patterns of fluorescence (nuclei and tubules) appear at the same time, but as these patterns are only visible in different focal planes it was not possible to combine the signals in a single micrograph.

As found with the peptide antibodies, no immuno-gold signal was obtained using the new anti-58 kDa serum in ultrathin sections of both infected protoplasts and tissue.

As for performance in immunogold labeling of the 48 kDa protein in transport tubules, the antiserum against *E.coli* expressed 58 kDa protein gave a better reaction than the pre-existing anti-48/58 kDa peptide serum. The gold label was obtained alongside the entire tubule (Fig. 2*c*) and not only at places where the tubules were broken (Fig. 3*c*).

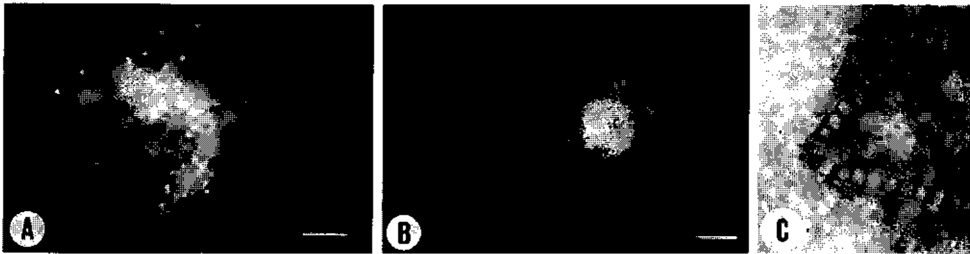



**Fig. 3.** Immunofluorescent images of infected cells treated with the peptide anti-48 kDa/58 kDa serum (*a*) tubular structures extending from the surface of the cell (*b*) fluorescence in nucleus. (*c*) Electron micrograph of a tubular structure immunogold labeled with the peptide antiserum against the 48 kDa/58 kDa proteins. Bars represent 5 μm (*a,b*) and 50 nm (*c*).

***Attempts to obtain antiserum specific for the 58 kDa protein***

In all immunological analyses sofar done, no discrimination between the 48 kDa protein and the 58 kDa protein could be made, due to the fact that antisera contained antibodies against their overlapping parts. Two approaches were tested to obtain an antiserum able to make this distinction. One approach was to synthesize peptides with sequences from the N terminus unique to the 58 kDa protein, and selected on their potentially high immunoreactivity. The other approach was to express the entire (10 kDa) N terminus of the 58 kDa protein in *E.coli*.

In the first approach two predicted antigenic regions of the 10 kDa N-terminal part of the 58 kDa protein were selected by computer analysis using the GCG program. Peptide 1 consisted of amino acids 33 to 57 and peptide 2 of amino acids 86 to 105 of the 58 kDa protein (for their exact sequence see Fig. 4).

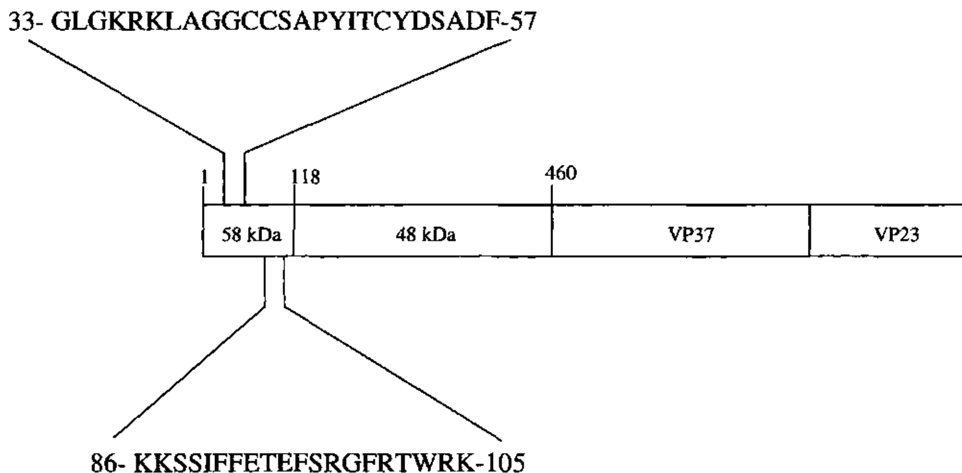



**Fig. 4.** Sequence and positions of the peptides used to raise 58 kDa specific antisera.

Antisera raised against these uncoupled peptides, reacted positively with the corresponding peptides, but not with the reciproke peptides, indicating their specificity (result not shown). Both antisera were then tested on *E.coli*-produced, purified 58 kDa on a Western blot using the pre-immune sera and the 58 kDa serum, raised against the *E.coli* produced 58 kDa protein, as a control. Both peptide antisera reacted with 58 kDa protein (result not shown) and were further tested on samples of infected protoplasts and on ultrathin sections of CPMV-infected leaf material. However, in these tests no reaction of either peptide antiserum was observed (results not shown).

As a second approach the entire sequence corresponding to the 10 kDa encoding N-terminal part of the 58 kDa gene was cloned into a pET vector and expressed in BL21 cells. However, upon SDS-PAGE and Westernblot analysis no 10 kDa peptide species was observed despite various repeated expression experiments, suggesting a very low stability of the expressed sequence.

***Immunolocalization of the 58 kDa protein of CPMV***
As no antiserum was obtained that would detect the 58 kDa specifically (i.e. without co-detection of the 48 kDa), the intracellular location of the 58 kDa protein was studied by transient expression in cowpea protoplasts. To this end cowpea protoplasts were transfected with vector pMon58 in which the entire 58 kDa open reading frame was expressed under the control of a CaMV 35S promotor. Controls consisted of mock-inoculated protoplasts or protoplasts transfected with the empty vector (pMon999). Protoplasts were analyzed by immunofluorescence microscopy using the antiserum raised against the *E.coli* expressed 58 kDa protein, at 24 h after transfection. Five % of the protoplasts transfected with pMon58 showed a bright staining of the nucleus (Fig. 5). The mock-inoculated and empty vector transfected protoplasts never showed this fluorescence confirming that the signal observed in the nucleus is caused by the 58 kDa protein.

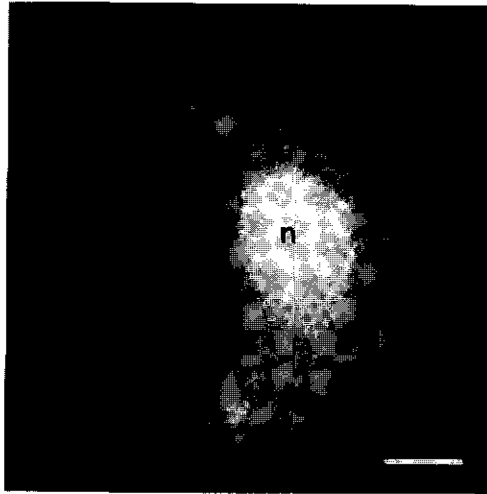



**Fig. 5.** Immunofluorescent image of a protoplast 24 h after transfection with pMon58 and treated with anti-58 kDa serum. Bar represents 5 μm.

## DISCUSSION

By transient *in vivo* expression of the 58 kDa protein it has now become clear that the 58 kDa protein of CPMV is not able to form tubular structures on its own, but rather that this protein is targeted to the nucleus. This was already suggested from previous experiments (Kasteel *et al.*, 1993, Chapter 2). The inability of the 58 kDa protein to form tubules on its own is a rather unexpected result as it contains the entire tubule forming domain of the 48 kDa protein. The presence of the extra N-terminal part of the protein must be responsible for this very different behaviour and for the targeting of this protein to the nucleus of protoplasts.

The nuclear localization of the 58 kDa protein was also previously observed (Kasteel *et al.*, 1993, Chapter 2) using a peptide antiserum against the overlapping 48 kDa/ 58 kDa proteins. This phenomenon has now been confirmed using a better antiserum, i.e. raised against the entire 58 kDa protein. This localization does not correspond with the essential involvement of the N-terminal domain of this protein in the replication of RNA 2 (Van Bokhoven *et al.*, 1993). It should be noted, however, that the nuclear localization has not yet been confirmed in protoplasts and tissue using immunogold electron microscopy. This might be caused by the fixation method used, which potentially destroys epitopes. Alternatively, the amount of 58 kDa protein in the nucleus might be below the detection limit of this method.

Several attempts were made to obtain 58 kDa specific antibodies that would allow discrimination between the 58 kDa and the smaller, overlapping 48 kDa protein *in vivo*. Antisera were made against peptides corresponding to computer predicted antigenic regions of the 10 kDa N-terminus of the 58 kDa protein and these could detect the 58 kDa protein on western blots. However, they failed to detect the protein *in situ*, possibly the epitopes are not

reachable in the native 58 kDa protein. From the computer analysis of the 58 kDa protein structure it is plausible that the 10 kDa N-terminus is not exposed, in view of its hydrofobicity, and this may cause a detection problem. Similar results have been obtained previously, using a peptide corresponding to the 30 N-terminal residues of the 58 kDa protein (Wellink *et al.*, unpublished results).

The alternative approach to produce 58 kDa specific antibodies, by expression of the sequence encoding the entire 10 kDa N-terminal part of the 58 kDa protein in *E.coli*, was not successful. No expression of this particular sequence was observed, indicating low stability.

The extra 10 kDa part, exclusive to the 58 kDa protein does not contain any obvious nuclear transport signals, but still seems responsible for the nuclear targeting and the inability of the 58 kDa protein to aggregate into tubules. Hence, this sequence may cause a very distinct conformation of the 58 kDa protein versus the (tubule-forming) 48 kDa protein. Besides the identified function in replication of the N-terminal part of the 58 kDa protein (Van Bokhoven *et al.*, 1993), it is still possible that the entire protein plays an additional role in the infection process, but all evidence obtained strongly suggest that this function is not related to virus movement.

*In planta* clouds of material have been found at the end of tubules that label with the peptide antiserum against the 48 kDa/58 kDa proteins (Van Lent *et al.*, 1990a). It seems that at this site aggregated 48 kDa protein is broken down after having performed its function (tubule formation and transport of the virus particles). Possibly also the 58 kDa is rapidly degraded. A speculative function of the 58 kDa protein therefore might be the capture of proteases that degrade the 48 kDa protein in a competitive way, so that tubules are not (all) broken down in the primary infected cell. Thus the 58 kDa would have a stabilizing effect on tubules and would indirectly propagate spread of infection to the next cell.

## ACKNOWLEDGEMENTS

We gratefully thank J.-C. Boyer and O. van Marle for their extensive contributions to this work. Also Joop Groenewegen is acknowledged for his technical assistance.

Chapter 4

# THE MOVEMENT PROTEINS OF COWPEA MOSAIC VIRUS AND CAULIFLOWER MOSAIC VIRUS INDUCE TUBULAR STRUCTURES IN PLANT AND INSECT CELLS

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## SUMMARY

The movement proteins (MPs) of cowpea mosaic virus and cauliflower mosaic virus (CaMV) are associated with tubular structures *in vivo* which participate in the transmission of virus particles from cell to cell. Both proteins have been expressed in plant protoplasts and insect cells. In all cases, immunofluorescent histochemistry showed that the MPs accumulate intracellularly as tubular extensions projecting from the cell surface. Additionally, electron microscopy revealed intracellular MP aggregates in CaMV MP-expressing cells. The data presented establish common features for the tubule-forming MPs: no other virus gene products are required for tubule formation and unique plant components (e.g. plasmodesmata) are not essential for tubule synthesis.

## INTRODUCTION

The cell-to-cell movement of plant viruses is associated with the modification of plasmodesmata mediated by the action of virus-encoded proteins called movement proteins (MPs). So far, two functional groups of MP have been identified (Maule, 1991; Lucas & Gilbertson, 1994). In one group, exemplified by tobacco mosaic virus MP, virus movement is mediated without overt changes in plasmodesmatal structure but with an increase in the plasmodesmatal size exclusion limit (Wolf *et al.*, 1989). Typically, viruses associated with this group are able to move from cell to cell in the absence of coat protein and therefore virions. In the second group, exemplified by cowpea mosaic virus (CPMV) and cauliflower mosaic virus (CaMV) MPs, electron microscopy suggests that the virions move through plasmodesmata and that the role of the MP is to modify these channels structurally through the formation of tubules that are able to accommodate virus particles (Maule, 1991, Lucas & Gilbertson, 1994). Using immunogold labeling, the MP has been identified as a structural component of the tubules for CPMV (van Lent *et al.*, 1990a), red clover mottle virus (Shanks *et al.*, 1989), tomato spotted wilt virus (TSWV; Storms *et al.*, 1995), grapevine fanleaf (Ritzenthaler *et al.*, 1995) and tobacco ringspot (Wieczorek & Sanfacon, 1993) nepoviruses and CaMV (Linstead *et al.*, 1988). However, the detailed compositions of the tubules and the mechanism for their formation have not yet been determined for any of these viruses.

Although the MP tubules are associated with plasmodesmata in infected tissue, they have also been seen as intracellular projections from the surface of infected protoplasts (van Lent *et al.*, 1990a; Perbal *et al.*, 1993; Storms *et al.*, 1995; Ritzenthaler *et al.*, 1995) lacking both a cell wall and plasmodesmata. In all cases the tubules exhibit a clear polarity extending from the surface of the protoplasts into the culture medium, thus mimicking the assembly of the tubule from the infected plant cell, through the plasmodesmata, into the cytoplasm of the neighbouring cells. This phenomenon has been used to determine whether other virus gene products are involved in tubule formation. Transient expression of the MP gene from CPMV (Wellink *et al.*, 1993) or TSWV (Storms *et al.*, 1995) in protoplasts resulted in tubule formation, thereby excluding a role for other virus gene products. In the case of TSWV, this was also shown after expressing the MP gene in *Spodoptera frugiperda* cells from a baculovirus vector where, surprisingly, surface tubules were again formed despite the cells being of non-plant origin (Storms *et al.*, 1995).

The MPs of CPMV and CaMV have been subjected to extensive mutational analysis (Wellink *et al.*, 1993; Thomas & Maule, 1995a,b) and there is some evidence (Thomas *et al.*, 1993) that they might have a related structure, despite the absence of significant amino acid sequence similarity. To extend the comparative analysis of these two proteins it was necessary

to establish whether the tubules made by the CaMV MP required other virus gene products for their formation, and to develop comparative expression systems. The CaMV MP has already been expressed in *Escherichia coli* (Citovsky *et al.*, 1991; Thomas & Maule, 1995b) and *S.frugiperda* cells using a baculovirus vector (Zuidema *et al.*, 1990; Maule *et al.*, 1992). The experiments presented here provide further evidence for a common function for tubule-forming MPs in that the CaMV MP similarly requires no other virus gene product for tubule formation and, like TSWV MP, both CPMV and CaMV MPs form tubules on the surface of insect cells. The latter phenomenon has provided the opportunity for a more detailed electron microscopy analysis of MP tubules.

## METHODS

### *Antisera*

A polyclonal serum recognizing the C-terminal 30 residues of the overlapping 48 kDa/58 kDa proteins of CPMV has been described before (Wellink *et al.*, 1987). A further polyclonal antiserum specific for the 58 kDa and 48 kDa proteins was prepared by injection of the CPMV 58 kDa protein expressed in *Escherichia coli* (J.-C. Boyer, unpublished results) into rabbits. Both antisera recognize the 58 kDa and 48 kDa proteins of CPMV. The two CaMV MP (P1) antisera used (Harker *et al.*, 1987; Maule *et al.*, 1992) have also been described before.

### *MP expression from recombinant baculoviruses*

For CaMV MP expression in insect cells, an *Autographa californica* nucleopolyhedrovirus (AcMNPV) recombinant expressing the CaMV (Strasbourg isolate) MP under control of the polyhedrin promoter (AcAM1; Zuidema *et al.*, 1990) was used.

To obtain a recombinant baculovirus expressing the CPMV (Nigerian isolate) MP from the AcMNPV p10 promoter, the sequence for the 48 kDa protein was cloned as a *BglII* fragment into the *BamHI* site of the transfer vector pAcAs3 (Vlak *et al.*, 1990) to give pAcJB1. Plasmid pAcAs3 contains p10 flanking sequences and a ß-galactosidase marker gene cassette. The *BglII* fragment was synthesized by PCR from plasmid pTM58S (Chapter 2, Kasteel *et al.*, 1993) using the primers, 5'GTATATTCTGCCCAGATCTGCCATGG AAAGCATTATGAGCC 3' and 5'CCGGGCAAACAAGATCTTAGCCTACGAA TTCTGTGGAAAAGCC 3'; the newly created *BglII* sites are underlined. The techniques used were essentially as described by Sambrook *et al.* (1989).

Recombinant viruses were obtained by co-transfecting *S. frugiperda* Sf21 cells with AcMNPV AcMO21 DNA (linearized with *Bsu*36I) and pAcJB1 DNA using the Lipofectin method as described by Groebe *et al.* (1990). The AcMO21 is a p10-based expression vector that contains a unique *Bsu*36I site in the p10 locus (Martens *et al.*, 1994). Recombinant viruses were selected as blue-coloured plaques upon addition of X-gal, and subsequently plaque-purified (Brown & Faulkner, 1977) to homogeneity.

For baculovirus expression, the *S. frugiperda* cell line IPLB-Sf21 (Vaughn *et al.*, 1977) was maintained as monolayers in Hink's medium (Hink, 1970) supplemented with 10 % (v/v) fetal bovine serum (FBS). Sf21 cells were infected with recombinant viruses at a m.o.i. of 5 $TCID_{50}$ per cell as described by O'Reilly *et al.* (1992) and incubated for 48 h or 64 h.

***Transient expression of CaMV MP in Arabidopsis protoplasts***

A vector for the expression of CaMV MP in plant cells was prepared by excising the *gus* gene from pSLJ4D4 (Jones *et al.*, 1992) using *Nco*I and *BamHI* and replacing it, after infilling of the 5' extension of the *Nco*I site using the Klenow fragment of *E.coli* polymerase I, with a PCR-derived copy of CaMV gene I. The gene I DNA fragment was adapted with the PCR primers to give a blunt end and *BamHI* site at the 5' and 3' ends, respectively. The resulting plasmid (pSLJ4D4gI) contained the complete MP coding sequence between the CaMV 35S promoter and octopine synthase 3' poly (A) signal.

*Arabidopsis thaliana* C24 protoplasts were isolated from 3-4-week-old plants grown on GM medium following the methods of Damm & Willmitzer (1988) and Karesch *et al.* (1991a). Protoplasts (0.75 x $10^6$) were treated with 10 µg of pSLJ4D4 or pSLJ4D4gI using PEG-mediated DNA uptake as described by Damm *et al.* (1989) and Karesh *et al.* (1991b). Treated protoplasts were incubated in darkness for 17 h at 26$^o$C. CaMV-infected protoplasts were isolated from infected Chinese cabbage plants as described previously (Perbal *et al.*, 1993).

***Protein analysis***

Insect cells or treated protoplasts were harvested, washed in fresh culture medium and the proteins analyzed by SDS-PAGE (Laemmli, 1970) in 10% polyacrylamide. After electrophoresis, gels were either stained with Coomassie brilliant blue or used for immunoblotting to nitrocellulose. For immunodetection, blots were blocked overnight with 5% (w/v) fat-depleted milk powder and 0.01% (v/v) NP40 in PBS pH 7.2, incubated with antiserum (anti-CPMV MP diluted 1 : 3000 or anti-CaMV MP (Maule *et al.*, 1992) diluted 1 : 1000) for 2 h, washed with PBS and further incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Tago) for 1 h. After washing the immunoblots were developed using NBT and BCIP (BRL life Technologies) as substrates. All incubations were done at room temperature.

***Immunofluorescence microscopy***

Sf21 cells were grown on sterile glass coverslips in small Petri dishes and infected with the recombinants AcCPMV-MP or AcAM1. As controls Sf21 cells were infected with AcMO21 parental virus, wild-type AcMNPV or buffer. At 48 h and 68 h post-infection (p.i.) the cells were fixed with acetone at -70$^o$C for 7 min, washed with PBS and blocked for 45 min with 1% (w/v) BSA in PBS. The cells were then incubated for 1 h with anti-CPMV MP or anti-CaMV MP serum (Harker *et al.*, 1987) diluted in 1% (w/v) BSA in PBS, then washed extensively before incubating with FITC-conjugated horse anti-rabbit antibody (Nordic). After three 10 min washes with PBS, the cells were covered with glycerol-PBS containing Citifluor (Agar) and examined using a Leitz Laborlux S UV microscope.

*A. thaliana* and Chinese cabbage protoplasts were fixed and immunostained using anti-CaMV MP serum (Maule *et al.*, 1992) as described by van Lent *et al.* (1991).

***Electron microscopy and immunogold labeling***

Negative staining and immunogold labeling of AcCPMV-MP- or AcAM1-infected insect cells was performed after incubation for 48 or 64 h as described for protoplasts by van Lent *et al.* (1991). These cells were also fixed with aldehydes, dehydrated and embedded in LR Gold (London Resin Company) and sections were immunogold-labeled with antibodies against CPMV or CaMV MPs and Protein A-gold complexes with 7 nm or 10 nm gold particles, as described by Van Lent *et al.* (1990b).

Additionally, AcAM1-infected insect cells were fixed with aldehydes and infiltrated with 2.3 M sucrose in distilled water for 16 h. These specimens were then cryo-fixed by immersion in liquid propane using a Reichert KF80 cryofixation unit. Cryo-sections were prepared in a Reichert Ultracut S equipped with a FCS cryo-sectioning chamber. Cryo-sections were immunogold-labeled as described before and/or stained by applying a thin film of a mixture of 0.5% methyl cellulose and 0.5% uranyl acetate in distilled water. Specimens were examined in a Philips CM12 transmission electron microscope.

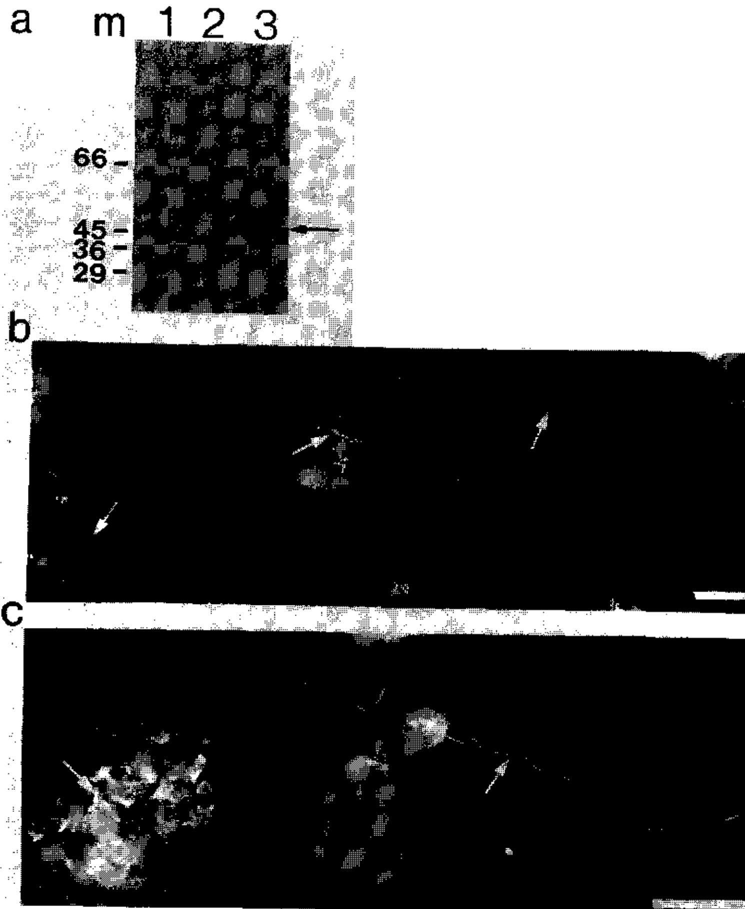


**Fig. 1.** Transient expression of CaMV MP in protoplasts. (*a*) Immunoblot of transiently expressed CaMV MP in *A. thaliana* protoplasts. Protoplasts were treated with control plasmid (pSLJ4D4; lane 2) or CaMV MP expression vector (pSLJ4D4gI; lane 3) and incubated for 17 h before analysis. Treatment with pSLJ4D4gI resulted in the accumulation of a 46 kDa polypeptide (arrow) that co-migrated with CaMV MP expressed in *E. coli* (lane 1).(*b*) Immunofluorescence assay of MP-expressing *A. thaliana* protoplasts showed MP-specific tubules (white arrows) extending from the surface of some protoplasts, similar to those formed after culture of pre-infected protoplasts isolated from CaMV-infected tissue (*c*). In (*a*) size markers were bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa) and carbonic anhydrase (29 kDa). Bars in (*b*) and (*c*) = 20 μm.

## RESULTS

***Transient expression of CaMV MP in plant protoplasts***

Previous work has shown that transient expression of CPMV MP in plant protoplasts results in the formation of tubules similar to those seen following virus infection of protoplasts, except that in the former case the tubules do not contain virus particles (Wellink *et al.*, 1993). To see whether the CaMV MP tubules could similarly form independently of any other virus protein, an expression vector containing CaMV gene I downstream of the CaMV 35S promoter was introduced into *A. thaliana* protoplasts. After treatment of protoplasts with this plasmid vector and incubation for 17 h the cells were harvested and assayed for the MP accumulation by immunoblot analysis and for tubule formation by immunofluorescent staining.

Immunoblot analysis with anti-CaMV MP serum (Fig. 1*a*) identified a range of polypeptides whether the protoplasts were treated with the control vector (pSLJ4D4) or the MP expression vector (pSLJ4D4gI). Most of these represented cross-reaction of the serum with host proteins but, in protoplasts treated with pSLJ4D4gI, a more intense band co-migrating at 46 kDa with *E. coli*-expressed CaMV MP indicated accumulation of expressed MP (Fig. 1*a*, lane 3).

Immunofluorescent staining of the protoplasts (Fig. 1*b*) showed that a small proportion of them (3%) displayed threads of fluorescence extending from the cell surface. Supported by electron microscopy evidence, we have previously interpreted these structures as tubules (Perbal *et al.*, 1993). The tubules were variable in length and similar in appearance to those seen in CaMV-infected Chinese cabbage protoplasts (Fig 1*c*).

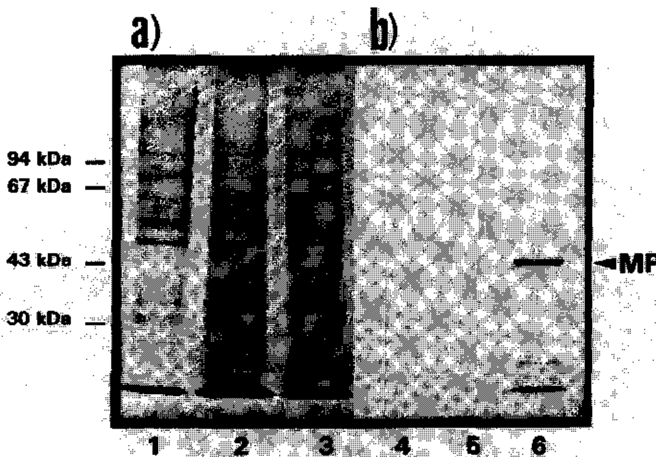


**Fig. 2.** SDS-polyacrylamide gel stained with Coomassie brilliant blue (a) and immunoblot analysis (b) of proteins extracted from uninfected (lanes 1,4), AcMO21- (lanes 2,5) and AcCPMV-MP (lanes 3,6) infected Sf21 cells at 48 h p.i. Immunoblot analysis was performed with the anti-CPMV MP serum prepared against the C-terminal 30 residues of the 58 kDa/48 kDa proteins of CPMV.

***Expression of CPMV MP by recombinant AcCPMV-MP***

Sf21 insect cells were infected with the baculovirus recombinant AcCPMV-MP, baculovirus AcMO21 or treated with buffer. After 48 h incubation, the total cell protein was analyzed by gel electrophoresis and immunoblotting. In cells infected with AcCPMV-MP a polypeptide was detected that was absent from cells infected with AcMO21 or buffer-treated cells (Fig. 2*a*). This protein co-migrated with CPMV MP present in CPMV-infected plant cells (data not shown) and was identified as the CPMV MP from immunoblots using the specific antiserum (Fig. 2*b*). A smaller polypeptide also reacted with the antiserum and probably represents partial breakdown of the MP.

Characterization of the expression of CaMV MP in AcAM1-infected insect cells has been reported previously (Maule *et al.*, 1992).

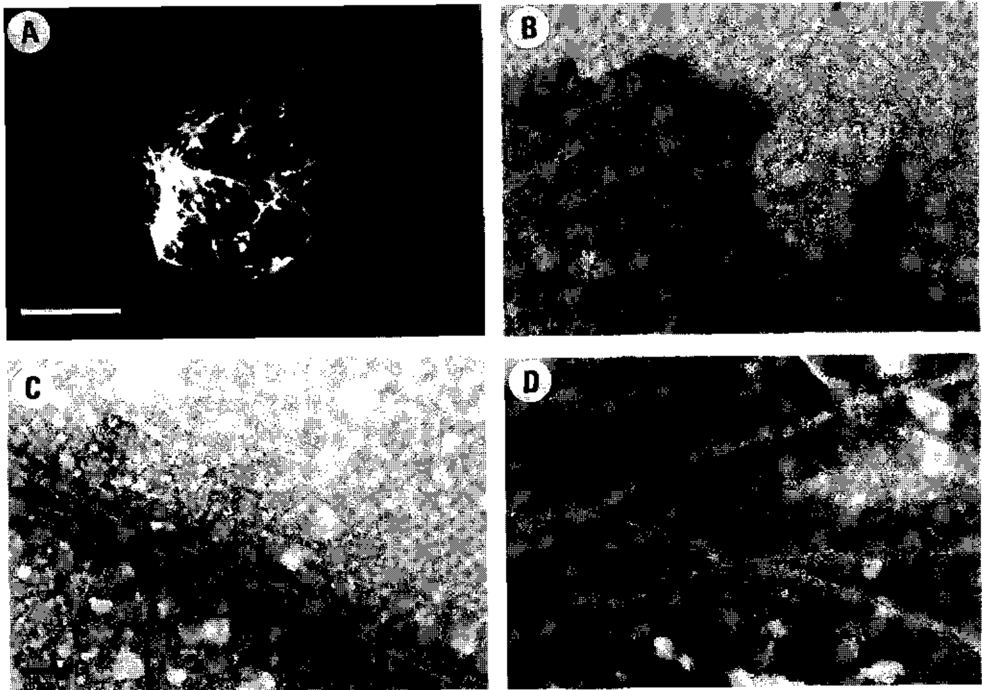


**Fig. 3.** Micrographs of AcCPMV-MP infected Sf21 cells at 48 h p.i. (*a*) Immunofluorescent image of an infected cell treated with the polyclonal antibody against the C-terminal 30 residues of the 58 kDa/48 kDa proteins of CPMV. Numerous tubules extend from the surface of the cells. (*b*) Immunogold localization of the MP in the cytoplasm along the plasmamembrane. (*c*) Negatively stained tubules found in whole mounts of infected cells and (*d*) immunogold labeling of these tubules after treatment with the anti-CPMV MP serum prepared against the entire 58 kDa protein of CPMV and Protein-A gold. Bars represent 10 μm (*a*) and 100 nm (*b, c, d*).

***Immunolocalization of the CPMV and CaMV MPs***

To study the subcellular location of the MPs of CPMV and CaMV in infected insect cells, Sf21 cells were infected with recombinants AcCPMV-MP and AcAM1 and incubated for 48 h and 68 h before preparation for immunofluorescence and electron microscopy. Cells infected with AcMO21, wild-type AcMNPV or treated with buffer were used as controls. In cells infected with recombinant AcCPMV-MP, immunofluorescent labeling with CPMV MP antiserum revealed numerous tubular structures extending from the surface of the cells into the culture medium (Fig. 3*a*).

Immunogold electron microscopy showed that in the cytoplasm, the CPMV MP was predominantly localized to the plasma membrane (Fig. 3b). In negative phosphotungstic acid (PTA)-stained whole mounts of AcCPMV-MP-infected cells, in which the cell structure was disrupted by an osmotic shock, many tubular structures were found (Fig. 3*c*) and immunogold labeling using the anti-CPMV MP serum identified the CPMV-MP as a component of the tubules (Fig. 3*d*). These tubules were the same diameter (35 nm) as those formed following expression of the MP in plant protoplasts and tissue (Van Lent *et al.*, 1991) and, as before, they appeared to lack any discernible contents. At higher magnifications no obvious substructure could be noticed. Occasionally the plasma membrane was observed along the tubular structure; it is likely that this membrane was often displaced during disruption of the cell for sample preparation.

In cells infected with recombinant AcAM1, immunofluorescent labeling with anti-CaMV MP serum similarly revealed the presence of numerous tubular structures emerging from the surface of the infected cell (Fig. 4*a*). The MP protein was also localized to large aggregates present in the cytoplasm of infected cells (Fig. 4*b*). Previously, these aggregates were reported to have a hollow fibre-like substructure (Zuidema *et al.*, 1990). Cryo-sections of AcAM1-infected cells revealed more detailed information on the substructure of these aggregates, showing them to have a 'rolled sheet' appearance (Fig. 4*c*). Negative PTA-stained whole mounts of the AcAM1-infected cells showed numerous tubule-like structures (Fig. 4*d*). At higher magnification these tubules revealed a detailed structure apparently consisting of an assembly of filaments (Fig. 4*e*) in a shallow twisted arrangement. The tubules had a mean (n=17) diameter of 52 nm with an average (n=7) of 14.7 filaments on the visible surface of each tubule. Immunogold labeling using the anti-CaMV MP serum identified the CaMV MP as a component of the tubules (Fig. 4*f*).

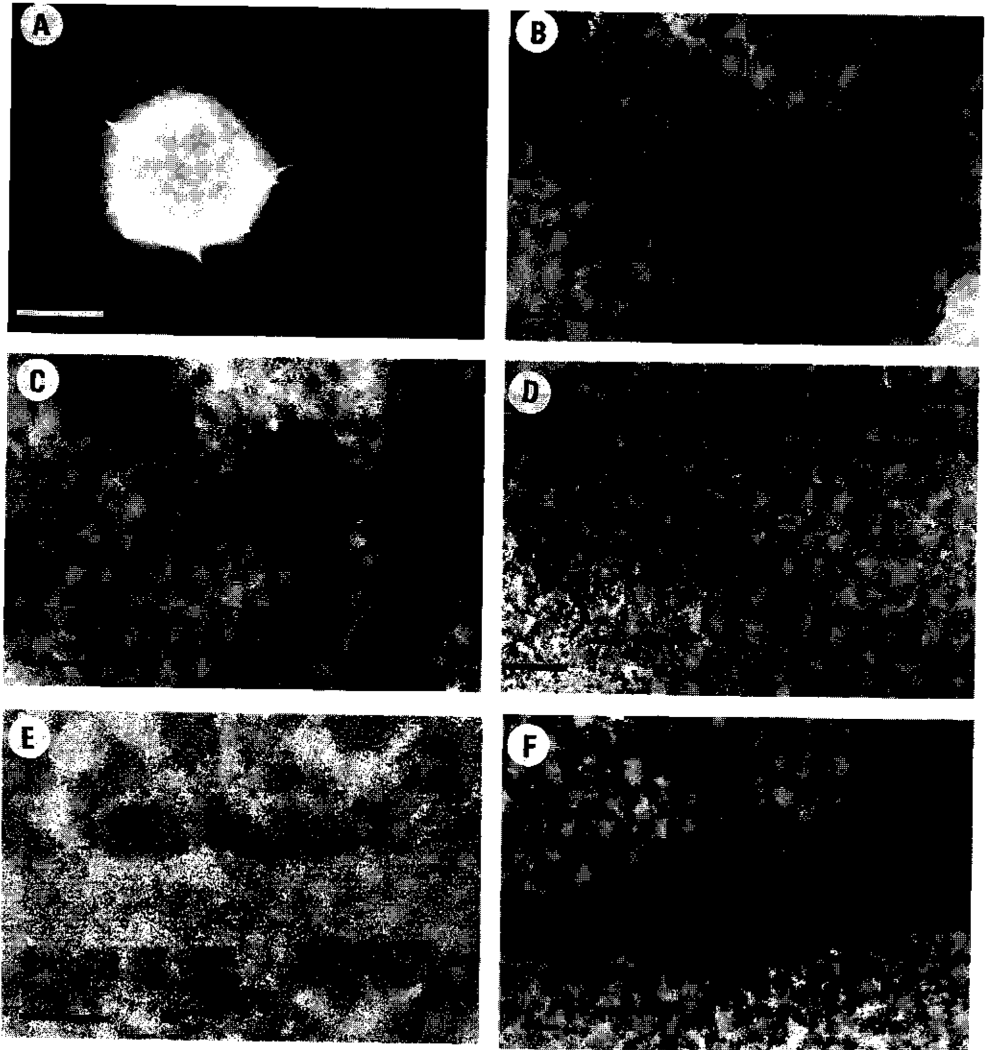


**Fig. 4.** Micrographs of AcAM1-infected Sf21 cells at 68 h p.i. (*a*) Immunofluorescent image of an infected cell treated with anti-P1 antibodies. Many tubules, some of which are branched, extend from the surface of the cell. (*b*) Immunogold localization of P1 in cytoplasmic aggregates and (*c*) a higher magnification of the cross-section of these aggregates showing the filamentous substructure. (*d*) Negatively stained tubular structures found in whole mounts of infected cells. (*e*) A tubular structure at high magnification revealing the filamentous substructure. (*f*) Immunogold localization of the P1 protein in tubular structures. Bars represent 10 μm (*a*), 50 nm (*b, c, e*) and 100 nm (*d, f*).

## DISCUSSION

The results of this study confirm that, like the tospovirus MP (Storms *et al.*, 1995), the MPs from the comovirus (CPMV) and caulimovirus (CaMV) groups are able to form tubules in the absence of other virus proteins and can achieve this in cells from outside the plant kingdom. This is despite these viruses having a different genomic organization and expression, and different virus particle size.

The observation that these MPs could form tubules extending from the surface of plant cells lacking a cell wall (protoplasts) could be accommodated in a model where the MPs recognised remnants of the plasmodesmata on the inner face of the plasma membrane as foci for the initiation of MP aggregation into tubules. However, the formation of similar structures at the surface of insect cells shows that either homologous target structures/proteins are present in the insect and plant kingdoms or that tubule formation is a spontaneous process when sufficient MP is present. The latter argument is less likely as MP tubules have not usually been seen free within the cytoplasm of plant or insect cells or extending into the plant cell vacuole.

The tubules formed in insect cells by the CPMV MP are morphologically identical to the tubules formed by this protein in plant protoplasts (van Lent *et al.*, 1991; Wellink *et al.*, 1993), exhibiting no pronounced substructure. In contrast, the tubules induced in insect cells by the CaMV MP contain a clear filamentous substructure and, in this respect, resemble the tubules formed by the TSWV MP protein. The tubules formed by the CaMV and TSWV MPs are also of similar dimensions (approximately 50 nm), a size capable of accommodating the respective nucleocapsids. The difference in surface structure between CaMV and CPMV tubules could be related to differences in the N termini of the proteins. Epitope tagging showed that the N terminus of CaMV MP may be exposed on the outer surface of the tubule, and structural alignments between the CaMV and CPMV MPs suggested that the latter could have an N-terminal extension with an α-helical structure (Thomas & Maule, 1995a,b).

Differences between the MPs were seen in their subcellular distribution in insect cells. The CPMV and TSWV (Storms *et al.*, 1995) MPs accumulated adjacent to the plasma membrane whereas the CaMV MP accumulated as large intracellular aggregates. The 'rolled sheet' appearance of the latter supports the view that tubules do not form spontaneously and accords with the observation from epitope tagging experiments in plant cells (Thomas & Maule, 1995a,b) that cytoplasmic CaMV MP has a different conformation from tubule-associated MP.

Although the MPs of CaMV, CPMV and TSWV show no sequence homology, an overall functional similarity (tubule formation) is apparent in that tubules are formed to transport virus particles from cell to cell. This concept is supported by computer analysis of the CaMV and CPMV MP sequences, which has revealed a similarity in the distribution of conserved and variable regions, suggesting a possible structural similarity between the two proteins (Thomas & Maule, 1995a,b). However, the difference in subcellular distribution may point to refined differences in the movement mechanism of these viruses.

In their natural hosts the MPs assemble in, what can be considered as a unique plant intercellular organelle, the plasmodesma (for review see Lucas & Gilbertson 1994). In view of the targeting of the MPs to and assembly of the tubules in these organelles, we must assume that specific intracellular cell trafficking mechanisms are involved. Despite the absence of plasmodesmatal components in insect cells, the high level of protein expression and abundant tubule formation for a diverse range of plant virus MPs make the baculovirus expression system an ideal way to make further progress in the analysis of the mechanisms of virus movement.

## ACKNOWLEDGEMENTS

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Chapter 5

# ISOLATION AND CHARACTERIZATION OF TUBULAR STRUCTURES OF COWPEA MOSAIC VIRUS

This chapter has been published in a slightly condensed form as: Kasteel, D.T.J., Wellink, J., Goldbach, R.W. and J.W.M. van Lent (1997). Isolation and characterization of tubular structures of cowpea mosaic virus. *Journal of General Virology* *78*, 3167-3170.

## SUMMARY

Tubular structures involved in the cell-to-cell movement of cowpea mosaic virus (CPMV) were partially purified from infected cowpea protoplasts to identify the structural components. A relatively pure fraction could be obtained by differential centrifugation and this was analyzed by PAGE and immunoblotting. Besides the movement protein (MP) and capsid proteins (CP) of CPMV, no other major infection-specific proteins could be detected, suggesting that host proteins are not a major structural component of the movement tubule.

## INTRODUCTION

Intercellular movement of CPMV is achieved by transport of virions through specialized tubules that are assembled in modified plasmodesmata. It was previously shown *in planta* that the CP and the 48 kDa MP of CPMV were located in these tubules (van Lent *et al.*, 1990a) and mutation or deletion of these proteins resulted in abortion of cell-to-cell movement (Wellink & van Kammen, 1989). In CPMV-infected cowpea protoplasts, the movement tubules occluding virions are extensively formed at the cell surface (Van Lent *et al.*, 1991), mimicking the process in plant tissue even in the absence of intact plasmodesmata. This material provides an opportunity for further identification and characterization of components involved in tubule formation. By analysis of deletion and insertion mutants and by transient expression experiments it was shown that the MP of CPMV was the sole viral protein responsible for tubule induction (Chapter 2, Kasteel *et al.*, 1993, Wellink *et al.*, 1993).

The typical association between the CPMV movement tubules and the plasma membrane (in protoplasts and in plant tissue) lead to the speculation that one or more host components could be involved, either as a structural component of the movement tubule or in the process of anchoring of the tubule at the plasma membrane. As expression of the MP by a baculovirus expression vector resulted in identical tubule formation at the insect cell surface (Kasteel *et al.*, 1996), it was suggested that host components, if involved at all in the tubule-forming mechanism, should be of a conserved nature (e.g. cytoskeleton proteins).

To identify the major structural components of the movement tubule, tubular structures were purified from CPMV-infected cowpea protoplasts (*Vigna unguiculata* 'California Blackeye') by means of differential centrifugation and analyzed for their protein content by gel electrophoreses and immunoblotting. As tubule isolation from intact plant tissue is not feasible, infected protoplasts were used for mass production of tubules. In protoplasts, these tubules protrude from the cell surface and are easy to separate from other cell constituents.

## METHODS

### *Infection and analysis of protoplasts*

Protoplasts were isolated and inoculated with CPMV RNA or mock-inoculated with water as described by Eggen *et al.* (1989) and screened for infection and tubule formation by immunofluorescence microscopy using polyclonal antisera against CP and MP (Wellink *et al.*, 1987), respectively. Immunofluorescence and negative staining electron microscopy of protoplasts and tubule fractions were carried out essentially as described by Van Lent *et al.* (1991). An important parameter for optimal tubule formation was the viability of isolated protoplasts, which was estimated by fluoresceine diacetate (FDA) staining as described by Power *et al.* (1990). A sample of 100 µl protoplast suspension was mixed with 2 µl of 5

mg/ml FDA in acetone for 2-4 min. Viable protoplasts were identified by fluorescence in a UV-microscope. In general, the number of tubules formed at the cell surface was related to the number of viable protoplasts in the isolated suspension, and therefore only suspensions with 98% or more viable protoplasts upon isolation were used in these experiments.

***Isolation of tubular structures***

Tubular structures were separated from protoplasts suspensions with more than 60 % infected cells and numerous tubules at 48 h after infection, by shaking for 20 min at 80 r.p.m. on a shaker. The protoplasts were then pelleted by centrifugation for 5 min. at 60 g. During this centrifugation the tubules remained in the supernatant, as was verified by immunofluorescence microscopy. This supernatant was collected and then tubules were pelleted by centrifugation for 10 min. at 15.000 g in an Eppendorf centrifuge. The pellet was resuspended in phosphate buffered saline (PBS), pH 7.2, and this partially purified fraction was analyzed by electron and immunofluorescence microscopy.

***Purification and analysis of tubule-containing fractions.***

Membrane remnants were removed by treating the fractions with NP40 for 1 h at 4°C. Then several partially purified tubule fractions were pooled and loaded on a sucrose cushion consisting of 1 ml 40 % (w/v) sucrose in PBS with 1% (v/v) NP40 in an Eppendorf tube and centrifuged for 5 min. at 15.000 g. The pellet was resuspended in PBS and the purity of the fraction was checked by immunofluorescence and electron microscopy. Subsequently, purified tubule fractions and similar fractions obtained from mock-inoculated protoplasts were analyzed by PAGE on a 10 % gel (Laemmli, 1970) that was either silver stained (Morrisey *et al.*, 1981) or used for immunoblotting.

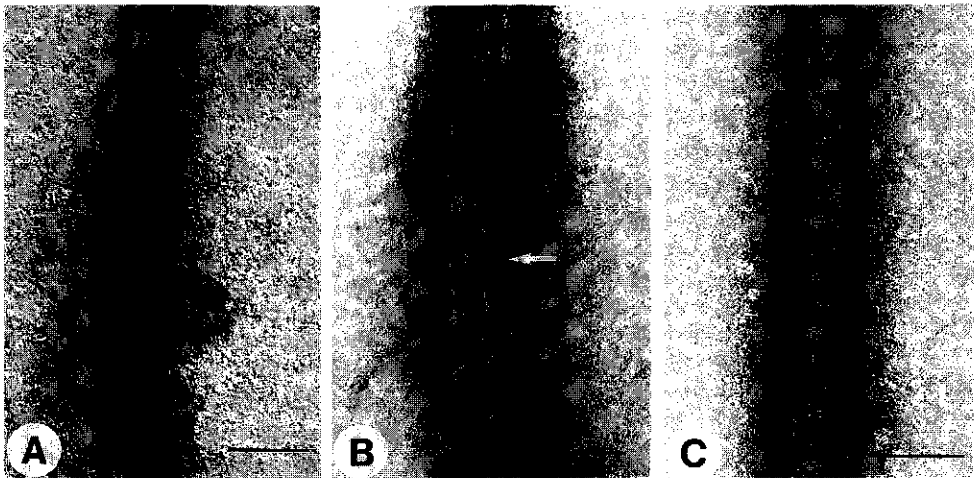


**Fig. 1.** Electron micrographs of tubules in partially purified fractions. Tubules are encased by the plasma membrane (a). After 3 weeks of refrigerated storage in PBS the tubular structure is still intact (b), but the plasma membrane has partially disintegrated (arrow). The plasma membrane was then successfully removed by treatment with nonidet P40 (c). Bars represent 100 nm.

## RESULTS AND DISCUSSION

The isolation of tubules from CPMV infected protoplasts resulted in a fraction containing numerous tubular structures of various lengths as observed in the electron microscope. Most of these tubules were still surrounded by a membrane (Fig. 1a). All tubules contained virus particles. The tubules in this partially purified fraction appeared to be stable for several weeks when stored in PBS at 4°C, but not at room temperature or frozen. After 3 weeks of storage in PBS only partial breakdown of the surrounding plasma membrane was observed (Fig. 1b). As this fraction still contained large amounts of cell debris, mainly chloroplasts, further purification was needed. Membrane remnants were removed using nonidet P40. This treatment did not have an apparent effect on the tubule structure (Fig. 1c).

Further purification resulted in a final fraction containing numerous clustered tubular structures (Fig. 2a and 2b) and very little cell debris when compared to the partially purified fraction. This fraction was analyzed by PAGE and immunoblotting.

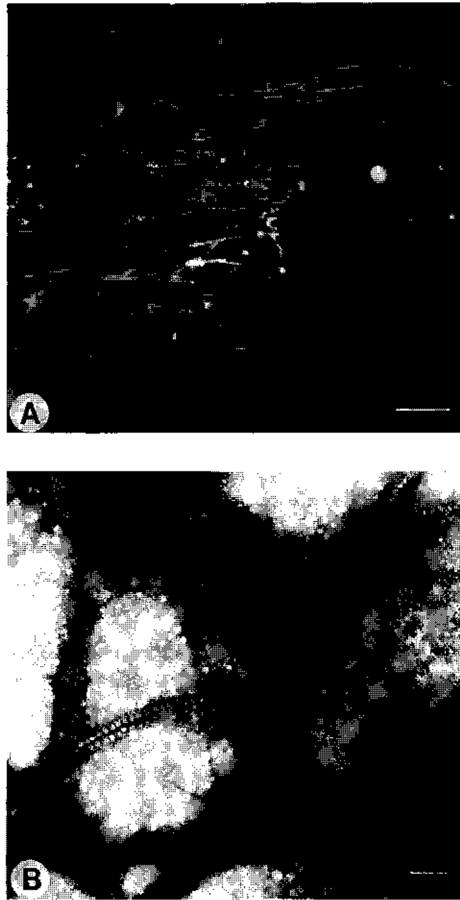


**Fig. 2.** a) Immunofluorescent staining of the pure tubule fraction, treated with anti-MP serum, showing numerous tubules. b) Electron microscopic image of negatively stained tubules in the same fraction. Tubules are mainly found in large clusters. Bar in (a) represents 10 μm, bar in (b) represents 100 nm.

The silver stained gel (Fig. 3a) showed three protein bands in tubule fractions that were absent in control fractions. These bands correspond to MP and the two CPs of CPMV as was verified on the immunoblot using anti-MP serum (Fig. 3b) and, subsequently, with anti-CP serum (Fig. 3c). The immunoblot shows an extra MP-specific band, of apparent molecular mass 36 kDa, which probably represents a breakdown product of the MP. Such a species also occurred in insect cells expressing the MP gene of CPMV (Chapter 4, Kasteel *et al.*, 1996). Apart from the MP and the CP, no other prominent infection-specific viral or host protein was detected in the partially purified tubule fraction.

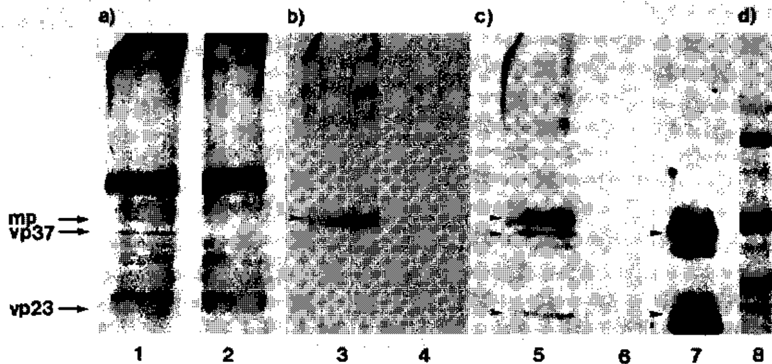


**Fig. 3.** Silver-stained SDS-polyacrylamide gel (a) and immunoblot analysis (b,c) of purified tubule fractions (lanes 1, 3 , 5) and similar fractions of mock-inoculated protoplasts (lanes 2, 4 , 6). Blots were treated with anti-MP serum (b) and subsequently with anti-CP serum (c). Arrows indicate the positions of the MP and the CPs VP37 and VP23. Lane 7 contains purified CPMV showing VP37 and a truncated form of VP23 (VP20) and lane 8 contains molecular mass markers.

These results support the hypothesis that the MP of CPMV is the only major structural component of the tubules. So far, only for cauliflower mosaic virus (CaMV) has an attempt been made to isolate and biochemically characterize tubular structures (Perbal *et al.*, 1993). Upon immunoblot analysis of a fraction obtained in a manner similar to that described here, only the MP of CaMV was detected. Further analysis of this fraction by SDS-PAGE followed by silver staining was not performed, presumably because of the low amount of tubules present in the fraction.

Although they are not a major structural component of the CPMV movement tubule, it remains to be determined if host proteins are in any way functionally involved in the process of tubule formation, e.g. in intracellular protein targeting or in anchoring of the tubule structure to the plasma membrane.

## ACKNOWLEDGEMENTS

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Chapter 6

# TUBULE-FORMING CAPACITY OF THE MOVEMENT PROTEINS OF ALFALFA MOSAIC VIRUS AND BROME MOSAIC VIRUS

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## SUMMARY

The structural phenotype of the movement proteins (MPs) of two representatives of the *Bromoviridae*, alfalfa mosaic virus (AMV) and brome mosaic virus (BMV), was studied in protoplasts. Immunofluorescence microscopy showed that the MPs of these viruses, for which there has been no evidence of a tubule-guided mechanism, assemble into long tubular structures at the surface of the infected protoplast. Electron microscopy and immunogold analysis confirm the presence of both MP and virus particles in the tubules induced by AMV and BMV. The significance of the tubule-forming properties of these viral MPs is discussed.

## INTRODUCTION

To establish systemic infection of plants, viruses modify the plasmodesmata to allow cell-to-cell movement of the virus particle or the viral genome (Lucas & Gilbertson, 1994). For a growing number of viruses it has been shown that one or more virus-encoded proteins are actively involved in this crucial process, and various biochemical activities have been assigned to these products.

Plant viruses can be categorized by their mechanism of intercellular movement. One category of plant viruses, including tobacco mosaic virus (TMV), does not require coat protein (CP) and moves in a non-virion form through the plasmodesmata. The virus-encoded MP is localized to the plasmodesmata (Tomenius *et al.*, 1987) and causes a significant increase in the size-exclusion limit, thus enabling the passage of viral RNA (Wolf *et al.*, 1989). Another category, including cowpea mosaic virus (CPMV), requires CP for intercellular spread and these viruses move as virions through tubules assembled within the plasmodesmata. In CPMV-infected cells, the viral MP is localized to these tubules (Van Lent *et al.*, 1990a). Typically, such movement tubules are formed not only in plant tissue but also in virus-infected protoplasts in the absence of cell walls and plasmodesmata (Van Lent *et al.*, 1991). A third category of plant viruses, including tobacco etch potyvirus (Dolja *et al.*, 1994) and potato virus X (Oparka *et al.*, 1996), also requires the CP for movement but tubule-guided virion transport through plasmodesmata has not been reported.

Recently, evidence was presented that two members of the *Bromoviridae*, alfalfa mosaic virus (AMV) and brome mosaic virus (BMV), also require CP for successful cell-to-cell movement (Van der Vossen *et al.*, 1994; Rao & Grantham, 1995). Although involvement of the AMV CP in cell-to-cell movement was established using CP mutants, it was not possible to conclude from these studies whether AMV moved as a virion or not. AMV mutant CPN199, with a C-terminal deletion in the CP, could move from cell to cell despite the fact that stable virions were not detectable (Van der Vossen *et al.*, 1994).

For BMV, Flasinski *et al.* (1995) concluded from their analysis of various CP-mutants that CP was not essential for cell-to-cell movement. However, Rao & Grantham (1995) showed that mutants which failed to produce an encapsidation-competent CP also failed to move from cell to cell and to induce local lesions, suggesting a requirement for CP for movement.

In view of these observations, we have investigated whether AMV and BMV also move as whole virions through tubule-like structures, by analyzing the phenotype of the respective MPs in cowpea protoplasts infected with these viruses.

## METHODS

### *Antisera*

Antibodies against the CPs of AMV or BMV were generated by injecting purified virus into rabbits. Antibodies against the MP of BMV (3a protein) were obtained by injecting a rabbit with *Escherichia coli*-expressed protein, and against AMV MP (P3 protein) as described previously (Van Pelt-Heerschap *et al.*, 1987).

### *Infection and analysis of protoplasts*

Cowpea protoplasts (*Vigna unguiculata* 'California Blackeye') were mock-inoculated with water or inoculated with AMV (strain 425) or BMV (strain M1) at a concentration of 10 μg virus per $10^6$ protoplasts, essentially as described by Eggen *et al.* (1989). Forty-two hours after inoculation, the protoplasts were analyzed by immunofluorescence and negative staining electron microscopy (Van Lent *et al.*, 1991) using antibodies against the respective MPs or CPs. Controls consisted of samples treated with pre-immune sera. Samples were analyzed using a Leitz Laborlux S fluorescence microscope and a Philips CM12 electron microscope.

## RESULTS AND DISCUSSION

At 42 h post-inoculation, an average infection of 60% (AMV) or 35% (BMV) of inoculated protoplasts was recorded. Approximately 75% of those protoplasts infected with AMV or BMV showed numerous fluorescent tubular structures at the cell surface upon staining with anti-MP sera (Fig. 1*a* and *b*). These tubules were also visible, though to a lesser extent, when anti-CP sera were used, indicating the presence of this protein in these structures (data not shown). Tubules were also observed on AMV-infected protoplasts from *Nicotiana benthamiana* and on BMV-infected protoplasts from *Hordeum vulgare* (not shown).

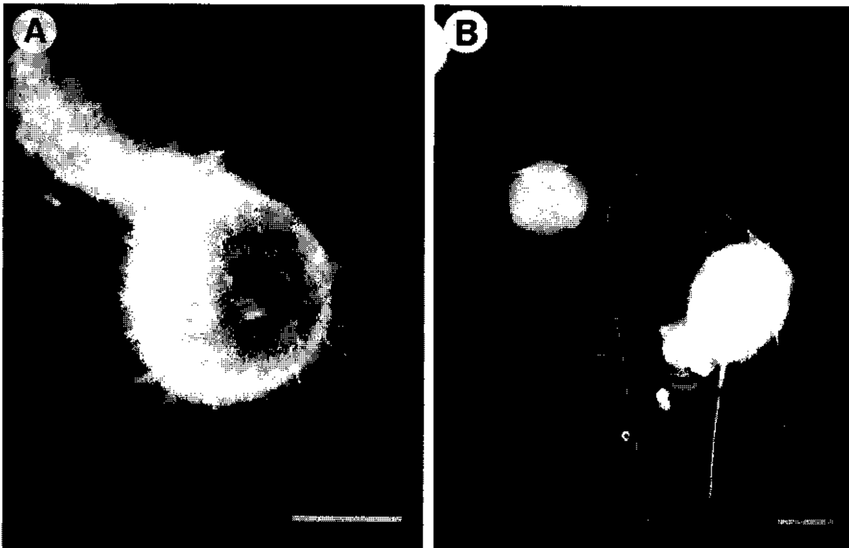


**Fig. 1.** Immunofluorescent images of cowpea protoplasts 42 h after inoculation with AMV (*a*) or BMV (*b*), labeled with homologous anti-MP serum. Bars represent 10 μm.

Electron microscopical examination of AMV- or BMV-infected protoplasts was performed by negative staining with 2% phosphotungstic acid (PTA) at either pH 5.5 or 6.5. These two pH values were chosen as BMV and AMV particles are stable at pH 5.5, but considerably less stable at pH 6.5. At pH 6.5, BMV particles swell and disintegrate (Johnson & Argos, 1985). In preparations stained with PTA at pH 5.5, virus-like particles were observed within the tubules induced by either virus (Fig. 2*b,c,e,f*). However, at pH 6.5 no such particles could be seen within the tubules or in the background (Fig. 2*a,d*), confirming that the structures observed at pH 5.5 indeed represented virions. The AMV- (Fig. 2*a*) and BMV- (Fig. 2*d*) infected protoplasts contained several tubules engulfed by plasma membrane. The phenomenon of multiple tubules enclosed by plasma membrane was also reported for CPMV (van Lent *et al.*, 1991). The average diameter of the tubules was 25 ± 3 nm and 40 ± 4 nm for AMV and BMV, respectively. BMV particles, with an average diameter of 31 nm, were neatly arranged in the wider tubule (Fig. 2*e,f*). The particle-like structures observed in the AMV tubules (Fig. 2*b,c*) had a diameter of 17 nm and resembled the icosahedral virions of AMV, but were less evident than BMV particles. Occasionally, in the AMV tubules, particles were observed that resembled bacilliform virions in size and shape.

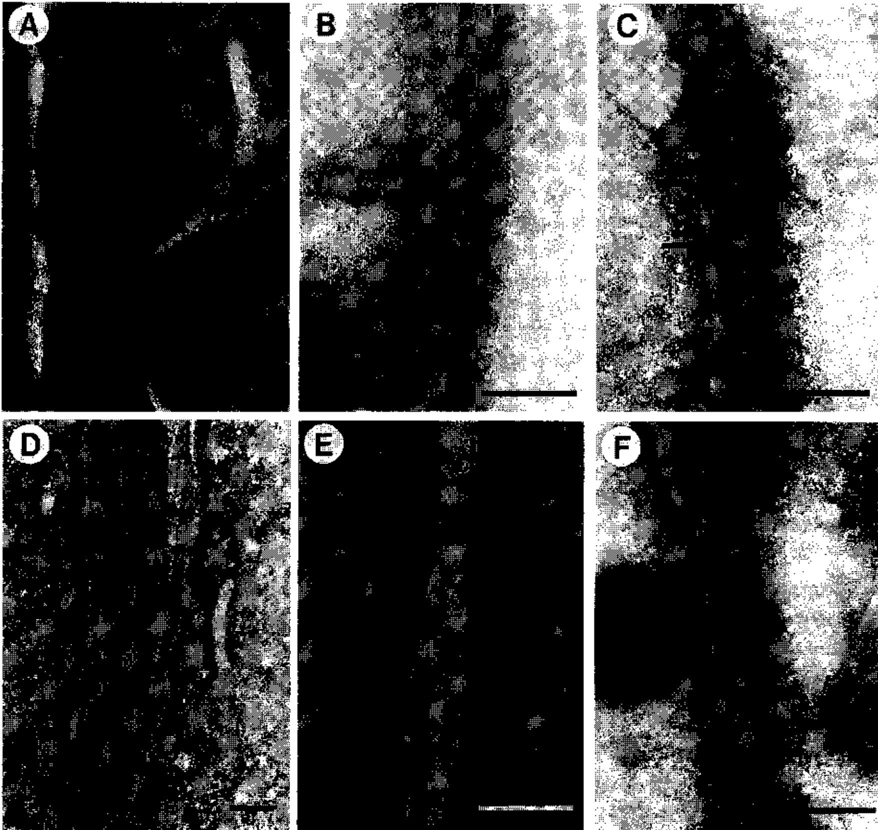


**Fig. 2.** Electron micrographs of tubular structures of AMV (*a,b,c*) and BMV (*d,e,f*), negatively stained with PTA at pH 6.5 (*a* and *d*) or pH 5.5 (*b,c* and *e,f*). Icosahedral and bacilliform (arrows) particle structures are visible within the tubular structure at pH 5.5. Arrowheads indicate the plasma membrane. Bars represent 100 nm.

The presence of AMV CP in the tubules was confirmed by immunogold labeling with anti-CP serum. Gold label was found along tubules, in particular at sites where the structure had partly disintegrated (Fig. 3*a*). Similarly, BMV tubules could be labeled with the homologous anti-CP serum (not shown). No gold labeling was found on intact BMV tubules, but gold complexes were found at places where virus particles were freely accessible (e.g. at the end of a tubule; results not shown). Apparently, the immunoglobulins cannot reach the CP antigen when tubules are intact, as has been found for CPMV tubules (Van Lent *et al.*, 1991).

The virus-induced AMV and BMV tubules could be labeled to a much better extent using the anti-MP sera (Fig. 3*b, c*), although in these cases the gold particles were also mainly found at sites where the structure of the tubules had partly disintegrated, thus exposing more antigen.

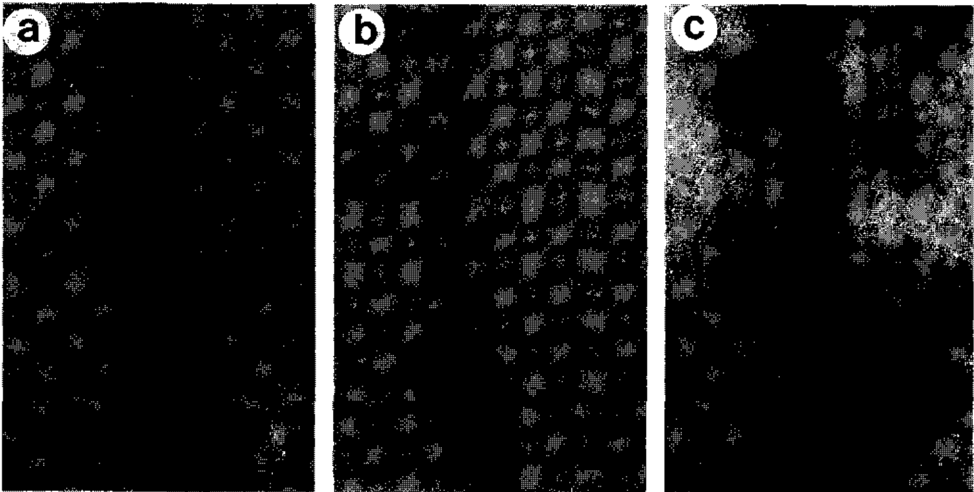


**Fig. 3.** Electron micrographs of immunogold-labeled tubules from AMV-infected (*a,b*) or BMV-infected (*c*) cowpea protoplasts and treated with anti-CP (*a*) or anti-MP sera (*b,c*). Bars represent 100 nm.

It is evident that AMV and BMV can induce tubular structures in infected protoplasts and that these tubules contain the MP and occlude virus-like particles. This complements the observations of van der Vossen *et al.* (1994) and Rao & Grantham (1995) on the requirement for CP for intercellular movement of AMV and BMV, respectively. Hence, by analogy with CPMV intercellular movement, it is plausible that movement of AMV and BMV particles through tubules assembled in plasmodesmata is a valid mechanism. In this respect, Godefroy-Colburn *et al.* (1990) noted the transient presence of tubule-like structures, gold labeled with anti-CP, in plasmodesmata of AMV-infected tobacco mesophyll parenchyma cells. Also, for members of two other genera of the family *Bromoviridae*, tobacco streak ilarvirus (Martelli & Russo, 1985) and tomato aspermy cucumovirus (Francki *et al.*, 1985), tubular structures containing virus particles have been observed in plasmodesmata of infected plant cells. However, the presence of virus-containing tubular structures in plasmodesmata of AMV- or BMV-infected plant tissue remains to be established.

## ACKNOWLEDGEMENTS

We thank B.J.M. Verduin for the antiserum against the BMV CP, P. Ahlquist for the BMV-MP serum and J.F. Bol for the antisera against the MP and the CP of AMV. We also thank J. Groenewegen for technical support, and J. Wellink and J.F. Bol for helpful comments during fruitful discussions. The research was supported by grants from the Foundation of Life Sciences (SLW, D.T.J. Kasteel, N.N. van der Wel) of the Netherlands Organization for Scientific Research (NWO) and the Joint Dutch-Israeli Agricultural Research Program (DIARP), grant number 93/29 (J.W.M. van Lent and R.W. Goldbach).

Chapter 7

# GENERAL DISCUSSION

## PROPERTIES OF THE 48 kDA MOVEMENT PROTEIN OF COWPEA MOSAIC VIRUS

Prior to discussing the results obtained during this thesis research, the conclusions of this work are summarized.
As described in Chapter 1 of this thesis, CPMV induces so-called tubular structures upon infection of a plant, which are implicated in cell-to-cell transport of the virus. The involvement of these structures in intercellular transport was on one hand inferred from immunogold-electron microscopy studies and on the other hand from deletion analysis revealing that the coding region for the tubule-forming 48 kDa protein is essential for viral cell-to-cell movement.

Tubular structures containing virus particles are not only induced in infected plant tissue, but surprisingly also on CPMV-infected protoplasts, i.e. single plant cells devoid of cell walls. In this thesis, this protoplast system was exploited to further investigate the involvement of both viral and possible host proteins in the induction and assembly of tubular structures.

Mutagenesis studies (Chapter 2) revealed that the region encoding the overlapping 58 kDa and 48 kDa RNA-2 encoded proteins is essential for tubule formation, but those of the capsid proteins not. However, from these studies it could not be discriminated whether each of these proteins is capable of inducing tubules or only one of them. Using transient expression vectors this was further investigated and it is now clear that the 58 kDa protein alone is not able to induce tubules in protoplasts (Chapter 3), whereas the 48 kDa protein is (Wellink *et al.*, 1993). The latter is also the only viral component found in fractions containing purified tubules (Chapter 5). The tubule forming capacity therefore seems to be an exclusive feature of the shorter 48 kDa polypeptide, which thus is the single movement protein (MP) of CPMV. In addition to the tubule forming capacity, the 48 kDa MP of CPMV contains a domain for the interaction with virus particles (Lekkerkerker *et al.*, 1996), and possibly additional domains, e.g. for targeting towards the periphery of the cell.

The 58 kDa protein appears to be essential for replication of the RNA-2 segment (Van Bokhoven *et al.*, 1993), a process which takes place in the cytoplasm of the infected cell. As the 58 kDa protein is also translocated into the nucleus (Chapter 2 and 3) it was suggested that the 58 kDa protein might have an additional role. To investigate this, attempts were made to obtain selective antibodies to this protein. However, these were not successful and therefore no evidence for a direct involvement of the 58 kDa protein in cell-to-cell movement could be obtained. Nevertheless a new antiserum directed against the entire 58 kDa protein was obtained, which will enable the study of for example 48 kDa/58 kDa C-terminal deletion mutants. Such experiments are not feasible with the peptide antiserum obtained before.

Besides the formation of tubules in plant tissue and in single protoplasts the MP is also capable of inducing tubules in insect cells, upon heterologous expression (Chapter 4). The latter finding implies that plasmodesmata (the proposed sites of tubule assembly in plants) are not necessary for tubule formation and that potentially involved host proteins should be of conserved nature (conserved between plants and insects).

Further research on the biochemical components of the tubules revealed that these movement structures only contain the 48 kDa protein (Chapter 5). No host proteins were detected. A structural role for host proteins in the process of tubule formation therefore does not seem very likely, but these could still be involved in a catalytic fashion. Candidate host proteins which are possibly involved in translocation of the MP, are the conserved cytoskeleton proteins, e.g. tubulin. For CPMV it has been suggested that microtubules, consisting of tubulin, play a role in the morphogenesis of tubules as they have been observed frequently as a lengthening piece of the tubules in infected plant tissue (Kim & Fulton, 1975). Alternatively, essential functions like for example the self-aggregation of MP into tubules, might well be encoded by the MP itself. As mentioned above, different domains have already been identified in the MP

| FAMILY | GENUS | GENOME | SPECIES | ABBRE-VIATION | TUBULES IN PLANTA | LOCALISATION OF MP TO TUBULES IN PLANTA | MP-CONTAINING TUBULES IN PROTOPLASTS | TUBULES IN INSECT CELLS |
|---|---|---|---|---|---|---|---|---|
| - | Caulimovirus | dsDNA | cauliflower mosaic virus | CaMV | +, 2,29 | +, 29 | +, 34 | +, 17 |
| | | | dahlia mosaic virus | DMV | +, 26,27 | | | |
| - | Badnavirus | | commelina yellow mottle virus | CoYMV | +, 1 | +, 1 | | |
| Geminiviridae | Geminivirus | ssDNA | euphorbia mosaic virus | EuMV | +, 24 | | | |
| | | | squash leaf curl virus | SqLCV | +, 48 | +, 48 | | |
| Reoviridae | Fijivirus | dsRNA | maize rough dwarf virus | MRDV | +, 9 | | | |
| - | - | - | black raspberry necrosis virus | BRNV | +, 14 | | | |
| Bunyaviridae | Tospovirus | ssRNA- | tomato spotted wilt virus | TSWV | +, 28,40 | +, 28,40 | +, 40* | +, 40 |
| Comoviridae | Comovirus | ssRNA+ | cowpea mosaic virus | CPMV | +, 42,44,45,20,23 | +, 44 | +, 45, 49*, 16 | +, 17 |
| | | | cowpea severe mosaic virus | CPSMV | | | +, 19 | |
| | | | red clover mottle virus | RCMV | +, 39 | +, 39 | +, 19 | |
| | | | bean pod mottle virus | BPMV | +, 20,21,22,23 | | | |
| | | | broad bean true mosaic virus | BBTMV | +, 30 | | | |
| | Nepovirus | | tobacco ringspot virus | TRSV | +, 4,47 | | | |
| | | | cherry leaf roll virus | CLRV | +, 46,47,15,3,37 | | | |
| | | | strawberry latent ringspot virus | SLRSV | +46,47,36 | | | |
| | | | grapevine fanleaf virus | GFLV | +, 35 | +, 35 | +, 35 | |
| | | | mulberry ringspot virus | MRSV | +, 13,41 | | | |
| | | | satsuma dwarf virus | SDV | +, 13,12,38 | | | |
| | | | tomato ringspot virus | ToRSV | +, 5,50 | +, 50 | | |
| | | | arabis mosaic virus | ArMV | +, 30 | | | |
| | | | tomato black ring virus | TBRV | +, 6 | | | |
| | | | myrobalan latent ringspot virus | MLRSV | +, 6 | | | |
| | | | artickoke yellow ringspot virus | AYRSV | +, 37 | | | |
| | | | olive latent ringspot virus | OLRSV | +, 7 | | | |
| Bromoviridae | Ilarvirus | | tobacco streak virus | TSV | +, 31 | | | |
| | | | citrus variegation virus | CVV | +, 10 | | | |
| | Cucumovirus | | tomato aspermy virus | TAV | +, 8 | | | |
| | | | cucumber mosaic virus | CMV | +, 46 | | | |
| | Bromovirus | | brome mosaic virus | BMV | | | +, 18 | |
| | Alfamovirus | | alfalfa mosaic virus | AMV | +, 43 | | +, 18,51** | |
| Sequiviridae | Sequivirus | | parsnip yellow fleck virus | PYFV | +, 33 | | | |
| - | Tymovirus | | brazilian eggplant mosaic virus | BEMV | +, 25 | | | |
| - | Tobamovirus | | tobacco mosaic virus | TMV | | | +, 19, 11** | |
| - | Umbravirus | ssRNA | carrot mottle virus | CMoV | +, 32 | | | |

**Table 1.**

* results obtained by transient expression of MP
** results obtained using a GFP-MP fusion protein

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49. Wellink *et al.*, 1993
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51. Zheng *et al.*, 1997

indicating that this single protein may display different biochemical activities to achieve the viral movement process.

## IS ASSEMBLY INTO TUBULES A MORE GENERAL PROPERTY OF PLANT VIRAL MPs?

When combining the results described in this thesis with additional experimental and literature data (Table 1), the picture emerges that the formation of tubular transport structures by plant viruses is a more general phenomenon than previously thought and that the cell-to-cell movement mechanism of CPMV may therefore be used as a model for many other viruses.

To date there is a considerable number of reports of viruses which form tubules *in planta*. For several of these viruses the MP has been localized to the tubules, underscoring the involvement of these tubules in the cell-to-cell movement process. Also, as a follow up of our research, quite a number of viruses have now been shown to form MP containing tubules in protoplasts. Even the ability to form tubules in insect cells is no longer a unique property of the CPMV MP; the MPs of TSWV and CaMV also assemble into tubules when expressed in insect cells (see Table 1 for references).

At the onset of this PhD research it seemed that the tubule-guided transport of virus was employed by a certain category of (como-like) viruses, whereas another category of (tobamo-like) viruses was suggested to use a different mechanism of transport. The results presented in Chapter 6 of this thesis, however show, that tubule formation in protoplasts also occurs for viruses which are genetically closely related to TMV, e.g. AMV and BMV. This was a surprising result and immediately gave rise to the question whether these viruses also form tubules *in planta*.

As for BMV, there is no evidence for tubule formation *in planta*, merely the observation of MP in plasmodesmata of infected plant tissue (Fujita *et al.*, 1998). Nevertheless, it may well be possible that upon closer examination of infected tissue of BMV, tubule-like modifications of plasmodesmata will be revealed. Not only because of the fact that BMV related viruses have been reported to form tubules containing particles in tissue (TSV; Martelli & Russo, 1985, TAV; Francki *et al.*, 1985), but also because of the fact that it is easy to overlook tubules in tissue as they are sometimes formed transiently and only at the infection front (TSWV; Storms *et al.*, 1995, AMV; Van der Wel *et al.*, 1998, see also below).

For AMV, modifications of plasmodesmata at the infection front have already been observed (Van der Wel *et al.*, 1998) and also tubule-like structures containing virus particles within the plasmodesmata (Godefroy-Colburn *et al.*, 1990, Van der Wel, personal communication). The finding of bacilliform AMV particles in tubules (Chapter 6) is rather surprising as formerly only round-shaped virions have been found in tubules. Recently, tubules containing bacilliform particles have also been observed for another virus, i.e. commelina yellow mottle badnavirus (Cheng *et al.*, 1998), confirming the possibility that non-spherical virions may use transport tubules as well.

The observation of tubule formation in protoplasts by MPs of viruses genetically related to TMV has given rise to the question whether the MP of TMV would also be capable of tubule formation in protoplasts, even though this virus has been defined as a virus which does not use a tubule-guided transport mechanism. Preliminary results (Fig. 1*a*) indicate that also TMV forms MP containing tubules in protoplasts. In contrast to the AMV and BMV induced tubules, these tubules do not contain virions (Fig. 1*b*), which is in conformity with the fact that TMV is translocated in a non-encapsidated form. The tubule forming capacity of the TMV MP in protoplasts has also been shown using a MP-green fluorescent protein (GFP) fusion protein (Heinlein *et al.*, 1998a). Still, no tubules have been observed in TMV infected plant tissue yet.

However, filamentous material consisting of MP was observed in plasmodesmata of TMV-MP transgenic plants (Ding *et al.*, 1992) and similar filamentous structures are also formed in the intercellular communication channels of prokaryotes (Heinlein *et al.*, 1998b).

It appears that the formation of MP-containing tubules in protoplasts occurs independently of particle shape, genome organization and taxonomic classification of plant viruses. This gives rise to the idea that tubular structure formation is an intrinsic property of plant virus MPs.

Although the tubule forming capacity seems a rather general feature of plant virus MPs, it is not clear whether this property is in all cases relevant *in planta*, in other words, whether a tubule is always necessary for transport of the infectious entity. If it is, then this means that in former electron microscopical studies of several viruses, tubules were not detected although being present. This could very well be the case, as the focus of electron microscopical studies performed was usually not on tubular structures while, moreover many aspects of tubule formation differ between plant viruses (e.g. their transient nature in some cases and formation at the infection front, see below) which could have been the cause of escaping our attention. A good example in this respect are the tubules of TSWV. TSWV infected plant material has been the subject of many electron microscopical studies in the past, however only in 1995 tubules were observed by Storms *et al.* It appeared that TSWV tubules are of transient nature and only occur at the infection front with a low frequency, thereby explaining why they were never seen before. Even for CPMV the ability to locate tubules has been shown to be dependent on the host studied; in *Vigna unguiculata* cv Early Ramshorn tubules were not detected (Langenberg & Schroeder, 1975).

The vast amount of data on tubular structures described in the reports quoted in table 1, show that tubules do not only differ in time and place of appearance, but also in many other aspects, e.g. contents and stability, diameter, frequency and probably also substructure, depending on the virus and host studied. All these factors determine whether a tubule *in planta* is easy to find or not.

Interestingly not only for TSWV, but also for some other viruses (CMoV, SqLCV, see table 1 for references) it has been described that tubules *in planta* can appear "empty", whereas most tubules described are filled with particles (as is the case for CPMV, CaMV and others, see table 1 for references). The finding of "empty" tubules (induced in protoplasts) for TMV (this Chapter) therefore is not a unique observation and it seems that transport of virus in forms other than mature virions via tubules (e.g. as nucleocapsid) could be a valid, but not extensively used, mechanism.

Of course until proven, it is still possible that formation of longer visible tubules is not necessary for the transport of the infectious entity, but that the association of the MP with the entity to be transported is enough to accomplish transport and that a lower aggregation form of the MP (such as the fibrillar structures in plasmodesmata observed for TMV (Ding *et al.*, 1992) and CMV (Blackman *et al.*, 1998) is also functional.

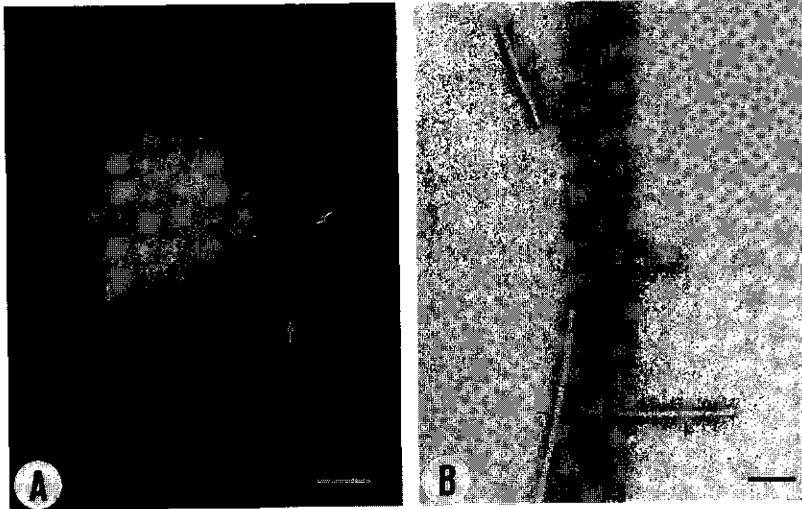


**Fig. 1.** *a*) Immunofluorescent image of a protoplast 48 h after infection with TMV treated with anti-MP serum. Tubular structures, extending from the cell surface, are indicated with arrows. *b*) Electron micrograph of negatively stained tubular structures of TMV. TMV particles are indicated with arrows. Bars represent 5 µm (*a*) and 100 nm (*b*).

## DO PLANT VIRUS MPs SHARE OTHER CHARACTERISTICS?

The idea that plant viral MPs act in a similar way is underscored by the fact that they have other properties, besides the tubule-forming capacity, in common. Namely, properties like the ability to change the gating capacity of plasmodesmata (as found for TMV; Wolf *et al.*, 1989, AMV; Poirson *et al.*, 1993, RCNMV; Fujiwara *et al.*, 1993, CMV; Vaquero *et al.*, 1994, PVX; Angell *et al.*, 1996, tobacco rattle virus; Derrick *et al.*, 1992), the ability to bind nucleic acids (as found for TMV; Citovsky *et al.*, 1990, 1992, CaMV; Citovsky & Zambryski, 1991, AMV; Schoumacher *et al.*, 1992, RCNMV; Osman *et al.*, 1992, Giesman-Cookmeyer & Lommel, 1993, CMV; Ding *et al.*, 1995, Li & Palukaitis, 1996, BDMV; Noueiry *et al.*, 1994, BMV; Jansen *et al.*, 1998, Fujita *et al.*, 1998) and the association to the cell wall/ plasmodesmata (as found for TMV; Tomenius *et al.*, 1987, CPMV, Wellink *et al.*, 1987, Van Lent *et al.*, 1990a, CaMV; Harker *et al.*, 1987, Albracht *et al.*, 1988, Linstead *et al.*, 1988, AMV; Stussi-Garaud *et al.*, 1987, Godefroy-Colburn *et al.*, 1986, Van der Wel *et al.*, 1998, TSWV; Storms *et al.*, 1995, RCMV; Shanks *et al.*, 1989, GFLV; Ritzenthaler *et al.*, 1995, geminiviruses; Pascal *et al.*, 1994, Sanderfoot *et al.*, 1996). All these properties occur for tubule-forming viruses and supposedly non-tubule forming viruses, thereby raising doubts about their relevance. The ability of viral MPs to increase the size exclusion limit of plasmodesmata has already been discussed by Storms *et al.* (1998), who showed that measuring this effect may also depend on the injection method used. Doubts about the functionality of the nucleic acid binding capacity of MPs exist because this ability has been established either by *in vitro* experiments or by *in vivo* micro-injection studies. For TMV the relevance of the nucleic acid binding capacity of the MP *in vivo* has been questioned by Kahn *et al.* (1998) based on mutagenesis studies.

As a result, besides a tubule-forming domain, domains for the interaction with virus particles (or nucleocapsids) and for targeting towards the periphery of the cell are expected in plant viral MPs. Mutational studies on MP encoding genes have indeed revealed such domains to be present (Berna *et al.*, 1991, Citovsky *et al.*, 1992, Gafny *et al.*, 1992, Thomas & Maule, 1995a,b, Erny *et al.*, 1992, Van der Vossen *et al.*, 1995, Giesman-Cookmeyer & Lommel, 1993, Osman *et al.*, 1991, Calder & Palukaitis, 1992, Lekkerkerker *et al.*, 1996, Kahn *et al.*, 1998).

In an attempt to unravel the mode of action of MPs, possible homologies of MPs to host proteins have been investigated, however only weak homologies are reported at the level of the primary sequence, e.g. with kinases (Saito *et al.*, 1988, Martinez-Izquierdo *et al.*, 1987), yeast cytochrome b apoprotein (Zimmern, 1983), plastocyanin (Hull *et al.*, 1986), maturases (Malyshenko *et al.*, 1989) and heat shock protein HSP90 (Koonin *et al.*, 1991). Another observed homology is the so called "D-motif" which appears to be present in all MPs (Koonin *et al.*, 1991, Melcher, 1990). Many attempts have been made to group MPs of viruses based upon functional and/or sequence homologies (e.g. Atabekov & Taliansky, 1990, Godefroy-Colburn *et al.*, 1990, Melcher, 1990, Koonin *et al.*, 1991). However, homologies of MPs of different viruses at the level of primary sequence is limited and often not significant. Extensive homologies at this level have only been reported for more closely related viruses. Only particular parts of the MPs show some conservation between related and unrelated viruses (Melcher, 1990, Koonin *et al.*, 1991). No homology exists between proteins of animal viruses and the plant viral MPs, indicating that animal viruses do not need a similar transport function (Goldbach, 1987, Atabekov & Taliansky, 1990). Other attempts to group the MPs of different plant viruses using different parameters (localization of the MP in the cell/plant, expression pattern of the MP gene in time and in different parts of the cell/plant, the ability to complement a defect in movement of another virus (Atabekov & Taliansky, 1990, Atabekov *et al.*, 1999), do not result in consistent classifications of these proteins.

A comparison of the tertiary structures would possibly give more information on the functional homology between MPs, however for such an analysis large amounts of soluble MP are necessary. The abundant production of MPs is not a problem as in the past several MPs have been expressed using different systems, e.g. insect cells, yeast and bacteria, however in all cases the MP obtained was insoluble and only would be dissolved at stringent denaturing conditions.

In view of the results presented in this thesis the protoplast-infection system seems a suitable system to study the characteristics of viral MPs. Though the protoplast infection system is an artificial system, it does rule out some of the factors that apparently influence the behaviour of the MPs in plants, e.g. host effects, the leaf age, symplast domains and physiological state. These factors have already been shown to influence aspects of MP localization/expression pattern. Therefore the cowpea/protoplast system seems a suitable approach when it comes to comparing MPs of different viruses and for example for the establishment of interactions of the MP with viral and/or host proteins during the process of translocation towards the plasma membrane. Results obtained with this particular protoplast system indicate that the translocation of the MP is similar for viruses that appear to be very different in movement mechanism when judged upon various analyses of the entire plant, e.g. AMV, BMV, TMV and CPMV. These MPs not only have in common that they form tubules at the surface of the protoplasts at a late stage of infection but also other fluorescent patterns observed with the anti-MP sera at earlier stages turned out to be similar. Rings of fluorescence, aggregates (small and large), threads, dots at the periphery have been observed for several of these viruses. Examples of intracellular MP patterns encountered are shown in Fig.2. Some of the patterns observed have also been described for TMV by Heinlein *et al.* (1998a) and Mas *et al.* (1998). The current research focuses on the determination of the order in which these patterns arise during the infection. This to understand the targeting of the MPs. At this point, the available data suggest that the MPs of plant viruses

have many characteristics in common and behave in a similar way although sequence similarity is limited.

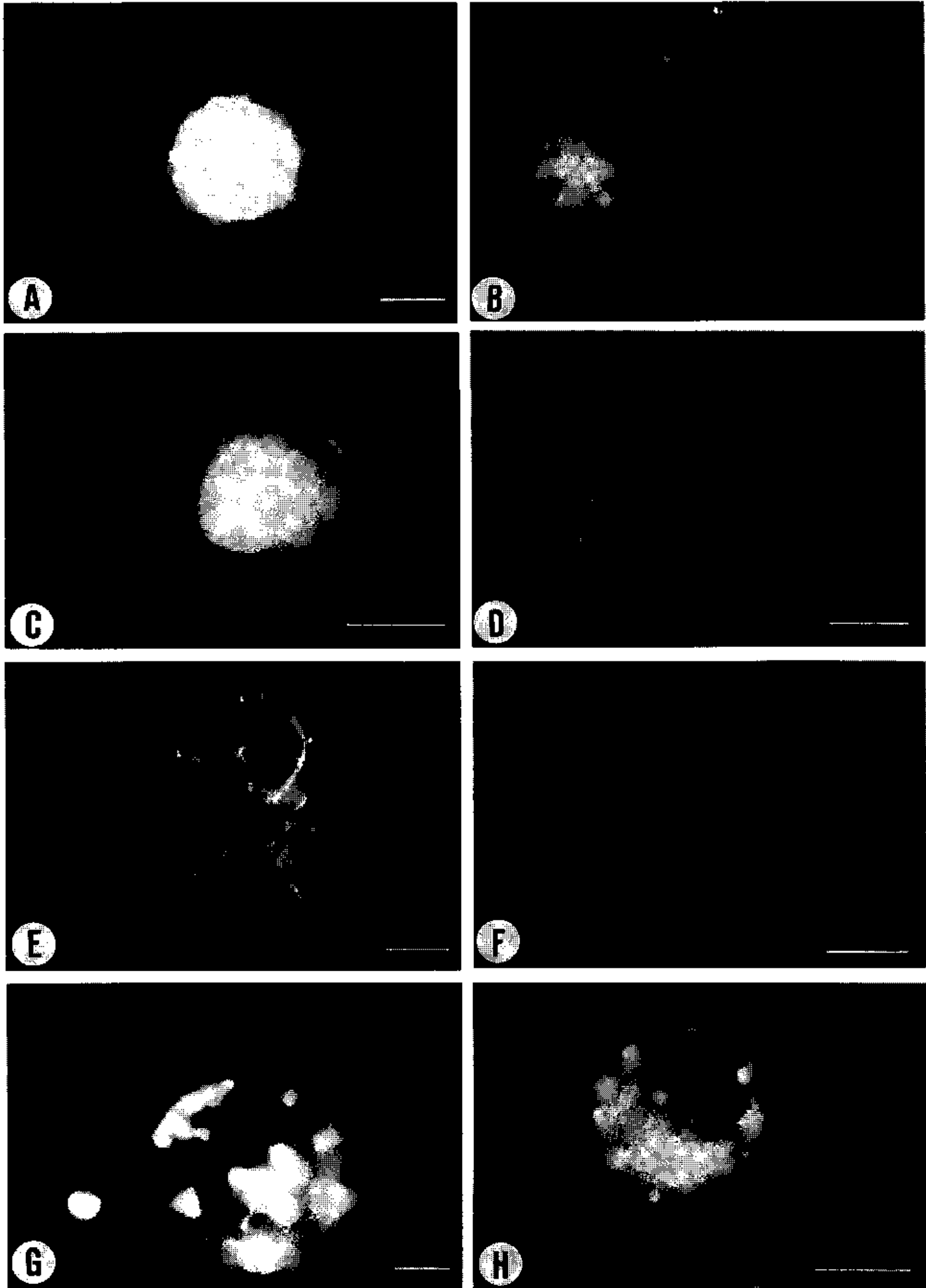


**Fig. 2.** Immunofluorescent images of protoplasts infected with CPMV and treated with anti-CPMV (*a*), AMV (*b,e*), BMV (*d,f,h*) or TMV (*c,g*) treated with the corresponding anti-MP sera. Bars represent 10 µm (*a,c,h*) or 5 µm (*b,d,e,f,and g*).

## MECHANISM OF CELL-TO-CELL MOVEMENT

The cell-to-cell movement of plant viruses is an intriguing subject, which gained a lot of attention, in recent years. Still the mechanism of transport of plant viruses is not well understood.

From the data described in this thesis it is clear that the two mechanisms described formerly, e.g. the (comoviral-type) movement as a virion via MP-containing tubules and the (tobamoviral-type) movement in a non-virion form via functionally modified plasmodesmata, are no longer sufficient to explain the movement of all plant viruses. It seems that the MPs of viruses which have been suggested to employ different movement mechanisms have many characteristics in common (see above) and this could mean that viruses move in principally the same way when looking at basic interactions and properties of the MP.

Combining the data described above it is possible to speculate on the various steps required to accomplish cell-to-cell transport and the components involved. The first step that is required is the association and subsequent transport of the infectious entity (virus particles/nucleocapsids or nucleic acid) towards the periphery of the cell. This step could be performed by the MP in combination with the cytoskeleton. Microtubules are suggested to target the TMV MP to the periphery of the cell, as an association of the cytoskeleton protein tubulin with MP has been observed (Heinlein *et al.*, 1995, 1998a, McLean *et al.*, 1995). Besides the cytoskeleton also the endoplasmic reticulum (ER) seems to play a role in MP targeting (SqLCV; Ward *et al.*, 1997 and TMV; Heinlein *et al.*, 1998a, respectively). Specific association of MP with virus particles has been established for CPMV (Lekkerkerker *et al.*, 1996) and associations between MP and viral RNAs have been established for several viruses among which there are tubule forming viruses (see above). Possibly during infection the MP of a given plant virus has affinity for both the viral nucleic acid and the CP and depending on the circumstances one or both will be bound by the MP, resulting in transport of virus particles and/or RNA. Whether the virus is transported as a particle or not depends maybe on other factors like the host, tissue, timepoint of infection etc. The latter theory possibly also explains why very closely related viruses like CCMV and BMV appear to use different movement mechanisms (e.g. transport of the viral genome and virus respectively, Rao, 1997). Such a difference could thus be the result of different hosts studied. It also explains the fact that for CMV, which has been suggested to traffic as a ribonucleoprotein complex containing the viral RNA, coat protein and MP (assembled into fibrillar formations, Blackman *et al.*, 1998), tubular structures containing virus particles have been observed occasionally (Walkey and Webb, 1968).

The second step would be the aggregation of MP into tubules or other (filamentous) forms that can facilitate the passage of the infectious entity through the cell wall. This aggregation most likely takes place at or near plasmodesmata as MPs have been observed near plasmodesmata frequently and also because sofar no MP in tubule form has been observed in the cytoplasm of protoplasts. The plasmodesmata as such do not seem necessary for tubule formation/aggregation (as apparent from their formation in insect cells, Chapter 4). There is evidence that in plant tissue plasmodesmata may be formed de novo at the infection front (Van der Wel *et al.*, 1998) pointing out the possibility that viral MPs stimulate plasmodesmatal biogenesis to create new openings in the cell wall for viral transport.

The third and last step would be the dissociation of the aggregated form of MP in order to release the infectious entity into the cell to be infected. This step requires a certain instability of tubules (or other aggregated forms of MPs). As described in Chapter 6 there are differences in stability/formation of tubules to be found in comparative experiments between different viruses. Such differences in stability could explain why tubules have not yet been observed for some viruses *in planta* whereas in the protoplast system they are readily formed. Viruses producing

less stable tubules could have an advantage as rapid breakdown of tubules in the cell to be infected results in a faster reléase of particles and/or nucleocapsids for replication and therefore a more rapid infection.

In conclusion, many aspects of cell-to-cell transport of viruses remain to be resolved, but it is clear from the results described in this thesis that there are more parallels between genetically unrelated viruses concerning this process than previously thought. Further studies on such parallels under standardized conditions will hopefully give us more clues about this intriguing subject.

# SUMMARY

## SUMMARY

During systemic plant infection, viruses move from the initially infected cells through plasmodesmata to neighbouring cells. Different mechanisms have been proposed for this cell-to-cell movement. Cowpea mosaic virus (CPMV) employs one of the major movement mechanisms, i.e. tubule-guided transport of virions, and this mechanism has been the subject of this thesis. The tubule-guided movement mechanism involves the assembly of movement protein (MP) into tubular structures within the plasmodesmal channel of infected cells, which pave the way for translocation of mature virus particles. These transport tubules are also induced on isolated plant cells (protoplasts) in the absence of cell walls and plasmodesmata. Employing this protoplast system, the structure, morphogenesis and function of CPMV tubules was studied.

By mutational analysis of RNA-2 of CPMV (Chapter 2) it was established that mutations in the coding region of the overlapping 48 kDa/58 kDa proteins, but not the capsid proteins, resulted in abolishment of tubule formation in protoplasts. Deletion of the capsid proteins resulted in the formation of tubules without virions. As the 58 kDa protein contains the entire 48 kDa sequence, mutations made in the overlapping coding region affect the function of both proteins. To establish the involvement of each protein in tubule formation, antisera specific to the unique 10 kDa N-terminus of the 58 kDa protein were made by using synthetic peptides. Although the antisera reacted to purified 58 kDa protein in immunoblots, they failed to react in immunocytochemical experiments. Alternatively, the 58 kDa gene alone was transiently expressed in protoplasts. In these protoplasts no tubular structures were formed and the 58 kDa protein apparently localized to the nucleus. As production of the 48 kDa protein in protoplasts was previously shown to result in tubule formation it was concluded that this protein constitutes the actual viral movement protein (MP). The possible function and significance of the 58 kDa protein are discussed in Chapter 3.

Having established that the 48 kDa protein is the viral MP responsible for tubule induction, the possible role of host proteins in this process was investigated following two different approaches. First, the expression of the MP gene in a heterologous (insect) cell system was studied (Chapter 4). As movement of plant viruses occurs through plasmodesmata, intercellular channels unique to plant cells, it was speculated that plant specific host factors could play a role in targetting and assembly of the tubules. However, upon production of the MP in insect cells, tubule formation occurred in a fashion similar to that in plant protoplasts. This led to the conclusion that if host proteins were involved in this mechanism, these should be of a conserved (among plant and animal) nature (Chapter 4). The second approach involved the isolation and subsequent biochemical analysis of tubules from infected protoplasts. This showed that the MP was (apart from the coat proteins) the sole major component of the movement tubules and host proteins were not obviously present in these structures (Chapter 5).

Tubule-guided movement of virions appears to be an important mechanism used by a large variety of plant viruses. For two other viruses which are genetically unrelated to CPMV, i.e. brome mosaic virus and alfalfa mosaic virus, evidence for this mechanism was obtained during the course of this PhD research. These two representatives of the Bromoviridae are genetically more closely related to tobacco mosaic virus (TMV) which supposedly does not move using a tubule-guided mechanism, but moves as an RNA-MP complex through plasmodesmata. The fact that the MP of these viruses also forms tubules in protoplasts indicates that tubule formation may be a more general ability of plant virus MPs (Chapter 6). This hypothesis is discussed and corroborated by literature data and additional experimental evidence in Chapter 7.

# SAMENVATTING

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Om infectie van een plant te bewerkstelligen moeten plantenvirussen zich van de initieel geïnfecteerde cel naar omliggende cellen en weefsels kunnen verspreiden. Voor transport van cel naar cel dienen virussen in staat te zijn om de rigide celwand te passeren en daarvoor maken zij gebruik van de plasmodesmata. Dit zijn cytoplasmatische verbindingen tussen naburige cellen die als al dan niet vertakte "kanaaltjes" door de celwand lopen en functioneren in de communicatie tussen cellen. De functionele diameter van deze plasmodesmata (ongeveer 3 nm) is echter vele malen kleiner dan de diameter van virussen of hun genoom. Voor het transport van cel naar cel door de plasmodesmata kunnen virussen gebruik maken van verschillende mechanismen. Eén belangrijk mechanisme van transport wordt gebruikt door het cowpeamozaïekvirus (CPMV) en vormt het hoofdonderwerp van het onderzoek dat in dit proefschrift is beschreven. Volgens dit mechanisme worden complete virusdeeltjes getransporteerd door speciale buizen die het virus in de plasmodesmata aanbrengt. Een belangrijk uitgangspunt bij dit onderzoek was dat dergelijke buizen ook kunnen worden gevormd in geïsoleerde plantencellen (protoplasten), die geen celwand of plasmodesmata bezitten. Met behulp van dit protoplastensysteem zijn de structuur, de morfogenese en de functie van de buisvormige structuren van CPMV bestudeerd.

Door het aanbrengen van specifieke mutaties in het RNA 2 van CPMV werd vastgesteld dat de regio coderend voor de overlappende 48 kDa/58 kDa eiwitten betrokken is bij de vorming van de transportbuizen in protoplasten en dat de manteleiwitten, die eveneens door dit RNA worden gecodeerd, hierbij geen rol spelen (Hoofdstuk 2). Verwijdering van de manteleiwitgenen uit RNA-2 (virusdeeltjes kunnen dan niet meer worden gevormd) bleek geen effect te hebben op de vorming van buizen, maar deze buizen bevatten uiteraard geen virusdeeltjes meer. Mutaties in de 48 kDa/58 kDa coderende sequenties leiden wel tot het verlies van het vermogen om buizen te vormen. Aangezien het 58 kDa eiwit de gehele 48 kDa aminozuur volgorde bevat, hebben mutaties in de RNA sequentie die codeert voor deze eiwitten een effect op het functioneren van beide eiwitten. Het was dus niet mogelijk om op basis van mutatieanalyse de afzonderlijk betrokkenheid van deze eiwitten bij het transportmechanisme vast te stellen. Echter door het 58 kDa gen afzonderlijk in protoplasten tot expressie te brengen kon worden vastgesteld dat het 58 kDa eiwit niet als structureel eiwit bij de buisvorming betrokken was, maar dat dit eiwit naar de kern werd getransporteerd. Deze waarneming was in overeenstemming met eerdere experimenten, waarin werd aangetoond dat het 48 kDa eiwit het enige virale genproduct is dat nodig is voor de buisvorming en derhalve het virale transporteiwit is (movement protein, MP). In Hoofdstuk 3 wordt de mogelijke functie van het 58 kDa eiwit verder bediscussieerd.

Het is aannemelijk dat naast het virale MP ook componenten van de plant een rol spelen bij het intercellulair transport van virussen. Het virus maakt tenslotte gebruik van plasmodesmata, structuren tussen de cellen die uniek zijn voor planten. Op twee manieren werd getracht inzicht te krijgen in de mogelijke rol van gastheereiwitten bij buisvorming en virustransport. Allereerst werd het MP gen tot expressie gebracht in dierlijke cellen (insectencellen). Naar verwachting zouden in dergelijke cellen geen buizen worden gevormd, omdat de plantspecifieke eiwitten die onderdeel uitmaken van de plasmodesma hierin ontbreken. Echter het MP was in deze insectencellen in staat om op dezelfde wijze transportbuizen te maken als was waargenomen in protoplasten. Dit betekent dat, indien gastheereiwitten betrokken zijn bij de vorming van dergelijke buizen, deze geconserveerd moeten zijn in zowel dier als plant (Hoofdstuk 4). Ten tweede werden buizen die in plant protoplasten werden gevormd, geïsoleerd en biochemisch geanalyseerd. Deze experimenten toonden aan dat alleen het MP onderdeel van de transportbuizen uitmaakt. Een katalytische rol van niet detecteerbare

hoeveelheden gastheereiwitten in het proces van buisvorming kon echter niet worden uitgesloten (Hoofdstuk 5).

Het mechanisme van transport van virusdeeltjes door buizen blijkt een belangrijke rol te spelen bij de infectie van soms zeer verschillende virussen. Zo werd dit mechanisme ook vastgesteld voor twee andere virussen, namelijk het bromemozaïekvirus (BMV) en het alfalfamozaïekvirus (AMV) (Hoofdstuk 6). Deze twee virussen behoren tot de familie van de Bromoviridae en zijn genetisch verwant aan het tabaksmozaïekvirus (TMV). Opmerkelijk genoeg vertegenwoordigt TMV een groep van virussen die een geheel ander mechanisme van cel-cel transport gebruikt, namelijk transport van een RNA-MP complex door morfologisch ongemodificeerde plasmodesmata. Het feit dat de MPs van BMV en AMV in staat zijn om te aggregeren tot transportbuizen zou kunnen betekenen dat dit een meer algemene eigenschap van plantenvirale transporteiwitten is (Hoofdstuk 6).

Deze hypothese wordt verder onderbouwd met behulp van gegevens uit de literatuur en additionele experimentele gegevens in Hoofdstuk 7. Dit hoofdstuk bevat tevens de algemene conclusies uit de resultaten van het promotieonderzoek.

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# NAWOORD

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Al bladerend door dit (bijna voltooide) proefschrift verschijnen er vele namen van mensen in mijn hoofd, die ik op deze plaats zou willen bedanken voor hun bijdrage. De vraag die dan vervolgens opdoemt is dan natuurlijk: **HOE ?!** Immers, alle namen vermelden is onmogelijk (ik heb namelijk maar twee pagina's gereserveerd voor dit nawoord... ) en slechts enkelen noemen is ook niet echt een oplossing. Ook had ik even géén idee **WIE** dan **WAAR** in het stuk aan bod diende te komen en **WAT** ik dan over deze of gene zou willen zeggen. Dit **"hoe-wie-waar-wat"** probleem is inmiddels opgelost. Ik heb bedacht dat ik het proefschrift maar als de rode draad ga hanteren, bladerend door de binnenkant en vervolgens de buitenkant en toebehoren beziend.

De **titelpagina's** laten denk ik duidelijk zien dat zowel Rob als Jan een grote bijdrage hebben geleverd, respectievelijk als zijnde mijn promotor en co-promotor. Dankzij hen heb ik de kans gekregen om dit promotieonderzoek op het laboratorium van Virologie te verrichten. Voor beiden geldt dan ook bedankt hiervoor en bedankt voor de begeleiding door de jaren heen. Rob, jou ben ik tevens erkentelijk voor je snelle nakijkacties en je altijd kritische blik. Jan, jij hebt natuurlijk de motivatie af en toe opgekrikt met je gezelligheid (vele koppen chocolademelk, lunches en zelfs golflessen). Ook heb ik van jou het "eerst denken, dan doen" principe afgekeken en toegepast (niet op alle vlakken overigens...). Verder ben ik je natuurlijk erkentelijk voor de grote mate van vrijheid die je me hebt gegeven. Wat betreft de hoeveelheid begeleiding tijdens mijn promotieonderzoek mag ik trouwens niet klagen, want behalve Jan en Rob zijn ook Ab en Joan zeer betrokken geweest hierbij. Ab, ik vond de discussies met jou altijd erg motiverend en wil je bedanken voor je voortdurend aanwezige interesse. Joan, bedankt voor je steun door dik (omvang meer dan 1 meter...) en dun (soms iets te dun...) en de vele discussies (ook over andere onderwerpen). Voor mij ben jij het levende bewijs dat directheid het langst duurt. Dit laatste is, geloof ik, niet echt iets wat van nature in mijn (Limburgse) genen aanwezig is, maar een eigenschap, die ik door jou zeer zeker heb leren waarderen. Ik heb me trouwens voorgenomen om vooral op deze plek "directheid" toe te passen....

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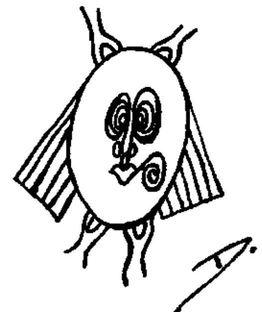
**BEDANKT ALLEMAAL!!!**

**Daniella**



# CURRICULUM VITAE

Daniella Theodora Johanna Kasteel werd geboren op 12 maart 1968 te Uden. In 1986 behaalde zij het V.W.O. diploma aan het Kruisheren Kollege te Uden, waarna zij biologie ging studeren aan de Landbouwuniversiteit Wageningen. In 1987 behaalde zij haar propedeuse diploma. In 1992 studeerde zij af met als hoofdvakken Moleculaire Biologie (Dr. J. Wellink) en Virologie (Dr. J.van Lent) en een stage Moleculaire Biologie bij Laboratoire de Virologie, Station de Pathologie Végétale, INRA-Bordeaux (Dr. O. Le Gall). In 1992 begon zij als onderzoeker in opleiding bij de vakgroep Virologie aan een door N.W.O. gefinancierd project. Dit onderzoek heeft geleid tot dit proefschrift. Sinds 1 september 1998 is ze als deelprojectleider werkzaam bij het Laboratorium voor Infectieziekteonderzoek (LIO) van het Rijksinstituut voor Volksgezondheid en Milieu (RIVM) en verricht ze onderzoek in het kader van het polio-eradicatie project.

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