THE COWPEA MOSAIC VIRUS MOVEMENT PROTEIN:

ANALYSIS OF ITS INTERACTIONS WITH VIRAL AND HOST PROTEINS

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Contents

CHAPTER 1

General Introduction

For successful systemic infection of a plant, a virus needs to overcome several barriers. It must enter and multiply in the initially infected cell and the progeny virus must spread from this primary infection site to the neighbouring uninfected cells (cell-to-cell movement) and to other plant parts (long-distance movement). Among plant pathogens, viruses are unique as they are no cellular organisms with their own metabolism, but are rather encapsidated parasitic (RNA or DNA) genomes, which fully depend on the host's cellular machinery for their replication. Viruses employ intercellular communication routes to move through the plant. For cell-to-cell movement, most, if not all, plant viruses use plasmodesmata to cross the rigid cell wall (Carrington *et al.*, 1996). Plasmodesmata are complex channels that span the cell wall and connect the cytoplasm of one plant cell with that of surrounding cells, and are crucially involved in intercellular communication (McLean *et al.*, 1997). For long-distance transport, viruses are proposed to use the vascular (phloem) system, following the same routes as plant assimilates and dependent on sink-source transition states (Carrington *et al.*, 1996, Santa Cruz, 1999). In all these transport processes, one or more viral proteins play an essential role. Most plant viruses have been demonstrated to encode a so-called "movement protein" (MP), which is actively involved in plasmodesma-mediated cell-to-cell transport of infectious entities (Lazarowitz, 1999; Lazarowitz & Beachy, 1999; Lucas & Wolf, 1999). The focus of this thesis is on the cell-to-cell movement mechanism of *Cowpea mosaic virus* (CPMV), a positive stranded RNA virus with spherical virion morphology.

Cell-to-cell movement of plant viruses

Detailed studies of the cell-to-cell movement mechanism of an array of plant viruses have revealed that most viruses encode one or more MP's that facilitate the transport of the virus through the plasmodesmata (for reviews see Carrington *et al.*, 1996, Citovsky & Zambryski, 1991; Lazarowitz & Beachy, 1999). As the size of the plasmodesma is too small for the passage of virions or even the naked viral genome, they employ a mechanism to modify these channels and allow their transport. Depending on the virus, plasmodesmata may be subtly or grossly modified. For instance, *Tobacco mosaic virus* (TMV) infection leads to an increased size exclusion limit (SEL) of the plasmodesmata without noticeable morphological changes to the plasmodesmal structure, while infection with other viruses, including CPMV, results in a complete structural transformation of the plasmodesma by the insertion of large

transport tubules. It can therefore be concluded that plant-infecting viruses have to encode a minimum of three types of proteins: the viral polymerase to multiply the viral genome, viral coat protein to encapsidate the progeny viral genomes, and the viral MP to achieve cell-to-cell movement (Mattheus, 1991).

Two main mechanisms for intercellular virus transport have been described: the movement of the viral genome in a non-encapsidated form, as exemplified by TMV (Deom *et al.,* 1987; Wolf *et al.,* 1989; Citovsky *et al.,* 1990; Waigmann *et al.,* 1994), and "tubule-guided" movement of mature virus particles (virions), as with *Cowpea mosaic virus* (CPMV) (Van Lent *et al.,* 1990; Kasteel *et al.,* 1993; Kasteel *et al.,* 1996). In the case of TMV it has been shown that its MP is able to bind single-stranded RNA (Citovsky *et al.,* 1990), to accumulate in plasmodesmata in infected cells (Tomenius *et al.,* 1987; Oparka *et al.,* 1997; Roberts *et al.,* 2001), and to increases the SEL of plasmodesmata to allow cell-to-cell transport of a viral ribonucleoprotein complex (Wolf *et al.,* 1989; Lucas & Gilbertson, 1994; Oparka *et al.,* 1997). Once this is done, the plasmodesma is compatible with the size and structure of the viral genome and this is able to move to the next cell (Citovsky *et al.,* 1990; Citovsky *et al.,* 1992). Prior to its interaction with the plasmodesma, the MP of TMV interacts with microtubules and actin filaments (Heinlein *et al.,* 1995; McLean *et al.,* 1995; Heinlein *et al.,* 1998; Lazarowitz & Beachy, 1999; Boyko *et al.,* 2000a), suggesting that the cytoskeleton plays an important role in targeting the viral genome/MP-complex to plasmodesmata. However, some authors have suggested that this association may be involved in targeting the MP for degradation during later stages of infection (for review see Tzfira *et al.,* 2000). Indeed it has been shown that the spread of some TMV mutants and the localization of their expressed MP is unaffected by disruption of microtubules, suggesting that the cell-to-cell movement of TMV is not dependent on microtubules (Gillespie *et al.,* 2002).

The second main type of movement, i.e. tubule-guided movement of virions, is employed by a broad variety of plant viruses, e.g. como- (Wellink & Van Kammen, 1989; Van Lent *et al.,* 1990; Van Lent *et al.,* 1991), alfamo- and bromo- (Kasteel *et al.,* 1997), nepo- (Wieczorek & Sanfacon, 1993; Ritzenthaler *et al.,* 1995), tospo- (Kormelink *et al.*, 1994; Storms *et al.,* 1995), caulimo- (Perbal *et al.,* 1993) and badnaviruses (Cheng *et al.,* 1998). In all cases the plasmodesma of infected cells is drastically modified, the desmotubules are removed and a transport tubule, assembled from the viral MP, is inserted into the plasmodesmal pore by which the virions are transported. Although most of the viruses that employ the tubule-guided movement mechanism to move cell-to-cell are icosahedral, virion shape and size does not affect assembly of MP into tubules or the subsequent movement of virions. Thus, *Tomato*

spotted wilt virus (TSWV; Storms *et al.,* 1995), which has enveloped particles, and bacilliform badnaviruses are also transported though tubules (Cheng *et al.,* 1998).

With most of the aforementioned viruses, infected protoplasts or protoplasts transfected with the MP coding gene exhibit the formation of tubular structures at their cell surface (Perbal *et al.,* 1993; Wellink *et al.,* 1993; Ritzenthaler *et al.,* 1995; Storms *et al.,* 1995; Kasteel *et al.,* 1996; Kasteel *et al.,* 1997). Even when expressed in insect cells, the MPs of CPMV, TSWV and *Cauliflower mosaic virus* (CaMV) retain their capacity to form tubules with a similar extracellular polarity (Storms *et al.,* 1995; Kasteel *et al.,* 1996). This implies that the MP alone is sufficient to induce the formation of the tubules and neither the CP nor plasmodesmata are required. Although it are the virions that are transported through the tubules, curiously the MP of some of the viruses that employ a tubule-guided mechanism also binds single-stranded nucleic acid *in vitro* (Citovsky *et al.,* 1991; Osman *et al.,* 1992; Schoumacher *et al.,* 1992; Fujiwara *et al.,* 1993; Jansen *et al.,* 1998; Qi *et al.,* 2002). In view of this, it has been proposed that CaMV and *Brome mosaic virus* (BMV) might use two alternative movement strategies to establish systemic infection: movement as a viral RNA-MP complex or movement as a complete virion by tubule-guided mechanism (Thomas & Maule, 1995; Jansen *et al.*, 1998).

As with TMV, bipartite geminiviruses move from cell-to-cell as nucleic acid without the requirement for CP. Geminiviruses have a DNA genome and multiply in the nucleus and for this reason they encode two distinct MPs (denoted NSP and MPB), one for nuclear export and the other for cell-to-cell movement, which co-operatively function in cell-to-cell movement (Sanderfoot & Lazarowitz, 1996). Geminiviruses are transported between cells as a viral DNA-MP complex by tubules that extend from and across the walls. NSP is targeted to the nucleus where it binds to viral ssDNA and transports the complex through the nuclear pore to the cytoplasm. Next, MPB transports the NSP-ssDNA complex to the cell periphery and cell wall. The MPB increases the plasmodesmal SEL and facilitates the movement of the DNA to the neighbouring cells (Noueiry *et al.,* 1994), probably by endoplasmic reticulum (ER)-derived tubules that cross the cell wall (Ward *et al.,* 1997). In the next cell the complex dissociates and the NSP transports the ssDNA again to the nucleus to initiate a new round of replication and infection (Sanderfoot & Lazarowitz, 1996).

A more complex, but less understood movement mechanism is employed by a diverse group of viruses, namely the potexviruses, hordeiviruses, furoviruses and carlaviruses. These all contain a cluster of three genes called triple gene block (TGB), encoding three proteins that are required for cell-to-cell movement (Beck *et al.,* 1991; Gilmer *et al.,* 1992; Petty *et al.,* 1990) and in some instances also require CP for intercellular transport. The functions of the TGB have been studied in most detail for

Potato virus X (PVX), which encodes proteins of 25, 12 and 8 kDa (Morozov *et al.*, 1997; Verchot *et al.*, 1998). The PVX CP localizes to the plasmodesma and during infection, the protein is transported between cells. However, studies in transgenic plants that express CP have shown no effect on the plasmodesmal pore (Oparka *et al.*, 1996; Santa Cruz *et al.*, 1998). Although the 25 kDa localizes in the cytoplasm in the form of inclusion bodies (Davies *et al.*, 1993), it can drastically increase the SEL of the plasmodesmata to facilitate the movement between cells, possibly in conjunction with the 12 kDa protein, whose function in movement is as yet unknown.

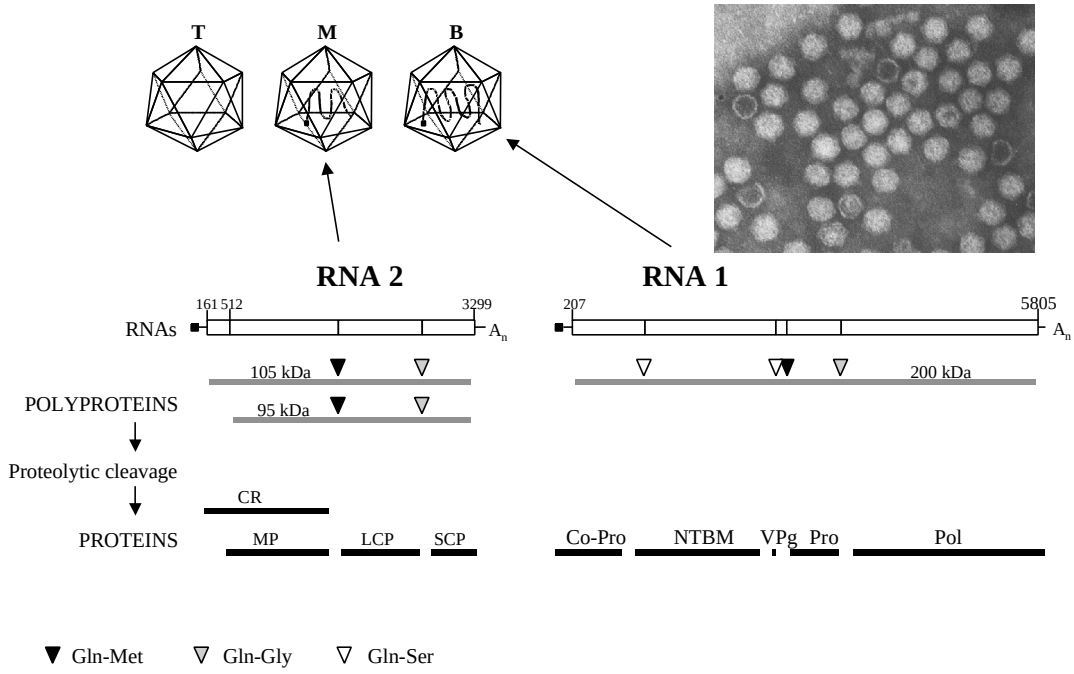

Figure 1 Organisation and expression of the CPMV genome. The positions of the start and stop codons on the RNA are indicated. RNA2 is translated into two polyproteins due to initiation of translation at position 161 and 512. Abbreviations: CR, cofactor for RNA2 replication; MP, movement protein; LCP, large coat protein; SCP, small coat protein; Co-Pro, cofactor for proteinase; NTBM, protein containing a nucleotide binding site motif; VPg, virion protein genome-linked; Pro, proteinase; Pol, RNA dependent RNA polymerase; The proteolytic cleavage sites are indicated by arrowheads. The micrograph shows negatively stained CPMV particles with a diameter of 29 nm. Virions suspensions contain a mixture of three particles, empty capsids (T) and particles containing the RNA2 (M) or RNA1 (B).

Cell-to-cell movement of CPMV

CPMV has been one of the model viruses in the study of tubule-guided cell-to-cell movement. Tubular structures were first identified in CPMV infected plants more than 30 years ago (Kim & Fulton, 1971; Van der Scheer & Groenewegen, 1971). CPMV is a member of the *Comoviridae*, genus *Comovirus* (Murphy *et al.*, 1995). Its genome consists of two single-stranded, positive-sense RNAs, denoted RNA1 and RNA2,

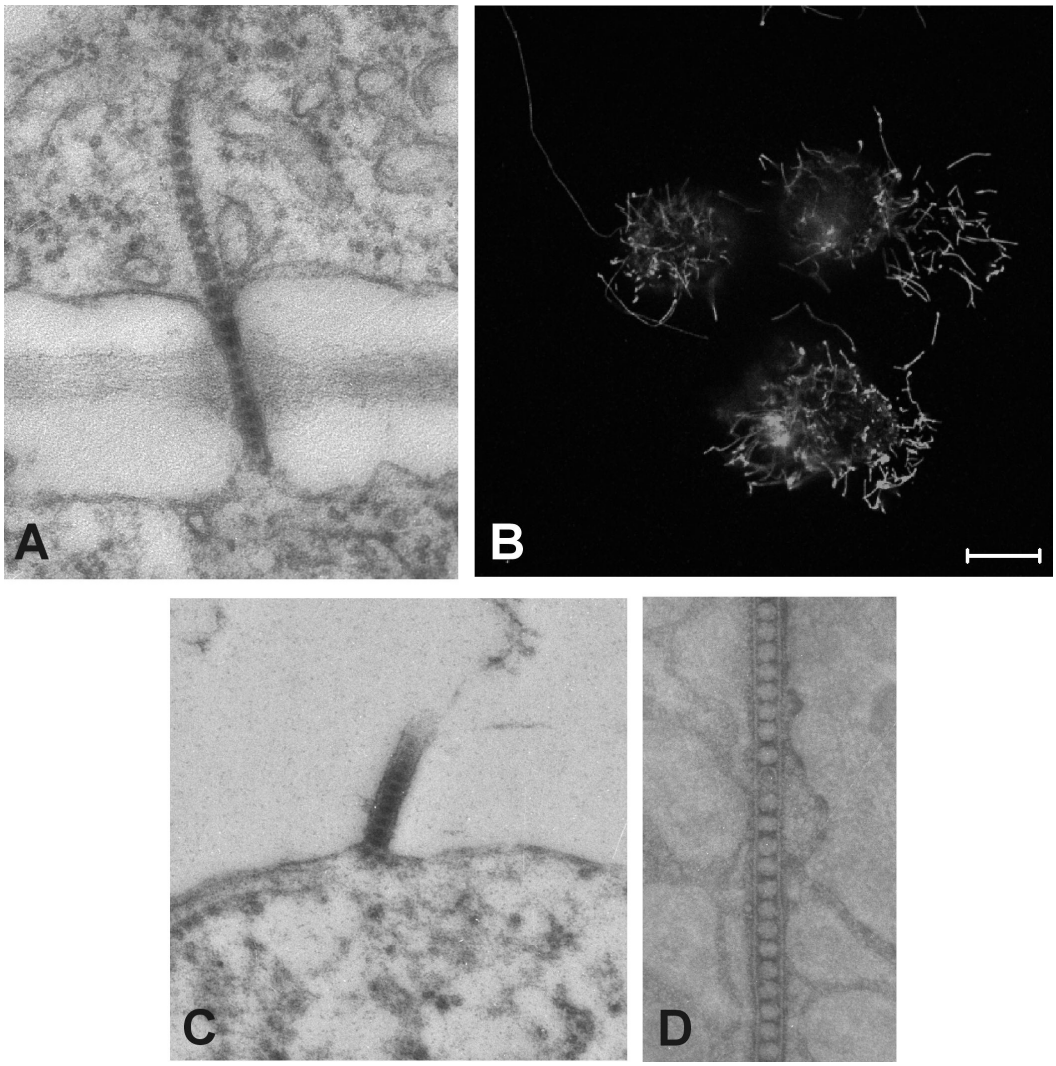

Figure 2 Transport tubules induced by CPMV in infected cells. (A) Electron micrograph of a tubular structure penetrating the cell wall though a modified plasmodesma. (B) Immunofluorescent image of a CPMV infected protoplasts, labelled with anti-MP serum. The tubules extend from the surface of the protoplasts. (C) EM micrograph of a tubule extending from the protoplast surface and (D) EM image of a negative stained tubule in a whole mount of infected protoplasts.

which are separately encapsidated in isometric particles of 28 nm in diameter (Fig. 1) (for review see Goldbach & Wellink, 1996).

The RNAs contain a small protein (VPg) attached to their 5'end and a poly-A tail at their 3'end. RNA1 codes for proteins involved in replication of the virus and this RNA is able to replicate in protoplasts in the absence of RNA2. RNA2 codes for two CPs (L and S), the 48kDa MP and a 58kDa cofactor required for replication (CR) of RNA2. Both MP and CP are necessary for cell-to-cell movement (Wellink & Van Kammen, 1989; Verver *et al.,* 1998). Although the CR contains the entire MP sequence, this protein is not able to induce tubule formation on its own (Wellink *et al.,* 1993).

In CPMV-infected plants, long tubular structures are present in modified plasmodesmata (Fig. 2A and Van Lent *et al.,* 1990). Immunogold labelling has shown

that the MP is a component of these tubules. Similar tubules are also formed in protoplasts infected with CPMV (Fig. 2B, C and D), where they grow close to the plasma membrane and extend into the medium (Van Lent *et al.,* 1991). These tubules are surrounded by the plasma membrane, suggesting that some host component may be involved in the tubule formation. However, the tubules are also formed in protoplasts from non-host plants upon expression of the MP (Wellink *et al.,* 1993) and even in insect cells transfected with the CPMV MP gene (Kasteel *et al.,* 1996), indicating that host components are not essential to tubule formation or, alternatively, these are strictly conserved between plant and insect species.

Mutational analysis of the CPMV MP has identified several functional domains (Bertens *et al.,* 2000). The C-terminus of the MP is involved in its proteolytic cleavage from the polyprotein precursor (Gopinath *et al.,* 2000) and this region is also essential for inclusion of virions during tubule assembly. MP devoid of this terminus is able to assemble into tubules, but these no longer contain virus particles (Lekkerkerker *et al.,* 1996). The N-terminal and central regions of the CPMV MP are involved in tubule-formation (Lekkerkerker *et al.,* 1996; Bertens *et al.,* 2000). Comoviral MPs do not show high homology among themselves. Sequence comparisons have shown that the most conserved residues are found in the central part, between residues 115 and 248. Inside this 33 amino acids-long area, there is some homology with the viral MP's that belong to the so-called 30 K superfamily (Mushegian & Koonin, 1993; Melcher, 2000). However, only one amino acid residue (the D motif) is strictly conserved (Koonin *et al.,* 1991; Mushegian & Koonin, 1993) and corresponds to residue 143 of the CPMV MP (Bertens *et al.,* 2000). Only one other motif, the so-called LPL motif, is also conserved in MP's outside the *Comoviridae* family. Mutational analysis has shown that this domain is essential for functionality of the comoviral MP in plants (Bertens *et al.,* 2000). The protein also contains a putative rNTP-binding domain (**L**-X-**D**-X-X-X-X-X-X-X-X-X-X-X-**V**-**A**-X-X-X-X-X-**G**-**R**), which is also conserved in the MPs of comoviruses (Chen & Bruening, 1992).

In contrasst to TMV, no clear involvement of host factors or organelles such as cytoskeleton elements or the endomembrane system has been reported for CPMV. However, it must be considered that neither MP nor the virions that are to be transported will reach the plasma membrane/plasmodesma simply by undirected diffusion. Furthermore, the external polarity of the movement tubules in protoplasts indicates a solid anchoring of the assembled tubule at the plasma membrane and thus to an association between one or more plasma membrane-residing factors and the MP. Once the cytoplasm of the neighbouring cell has been reached, the tubule must destabilise and release the virions. In all steps, host factors may play an essential role.

Host components involved in movement

The clearest functional relationship between virus movement and host factors has been reported for TMV, where the MP appears to interact with microtubules and F-actin in infected cells suggesting that these cytoskeleton elements are involved in the intracellular targeting of the MP-RNA complex to plasmodesma (Heinlein *et al.,* 1995; McLean *et al.,* 1995; Padgett *et al.,* 1996; Heinlein *et al.,* 1998; Lazarowitz & Beachy, 1999; Mas & Beachy, 1999; Boyko *et al.,* 2000a; Boyko *et al.,* 2000c). It has been suggested that a conserved tobamovirus MP sequence with similarity to a tubulin motif is the region that mediates the association of MP with microtubules during cell-to-cell movement (Boyko *et al.,* 2000b). Although the association of MP with microtubules suggests that these play a role in cell-to-cell movement of the viral RNA, movement of TMV can occur independently of microtubules (Gillespie *et al.,* 2002) and the microtubules may be involved in targeting the MP for degradation during the latter stages of infection rather than targeting for transport to neighbouring cells (Padgett *et al.,* 1996; Reichel & Beachy, 1998; Mas & Beachy, 1999; Gillespie *et al.,* 2002;).

Pectin methylesterase (PME) was shown to interact with the MPs of TMV, CaMV and *Turnip vein clearing virus,* and to be localised to the cell wall around plasmodesmata (Dorokhov *et al.,* 1999; Chen *et al.,* 2000). PME plays a role in cell wall dynamics (turnover and porosity) and has been implicated in plant responses to pathogen attack (Markovic & Jornvall, 1986). Deletion of the methylesterase binding domain in TMV MP aborts cell-to-cell movement (Chen *et al.,* 2000).

Yeast two-hybrid and Far-Western screens have identified host factors that show affinity to MP, but in most cases the function of these factors in the movement process is not at all understood. Matsushita *et al.,* (2001) reported a putative transcriptional co-activator (KELP, a protein that modulates host gene expression during pathogenesis) to show affinity for the MP of *Tomato mosaic tobamovirus*.

Also for viruses that employ a tubule-guided movement mechanisms, host factors with affinity for the MP have been found. In a two-hybrid screening with the MP of TSWV, Soellick *et al.,* (2000) found interactions with DnaJ-like chaperones. These proteins have functions including protein transport in organelles and the regulation of the chaperone Hsp70 (Kelley, 1999). It was additionally reported that TSWV MP is capable to bind proteins with homologies to myosin and kinesin, suggesting an involvement of molecular chaperones in the attachment of TSWV nucleocapsids to the cytoskeleton for subsequent intracellular trafficking (Von Bargen *et al.,* 2001).

Two-hybrid screening with CaMV MP revealed affinity for an Arabidopsis protein MPI7. The protein was localised to punctate spots at the cell periphery, probably representing plasmodesmata, and *in vivo* association between the MP and MPI7 was confirmed by fluorescence resonance energy transfer (FRET) (Huang *et al.,*

2001). In sequence, MPI7 is related to mammalian rab acceptor proteins, a family of proteins involved in protein transport through the endomembrane system and probably associated with the ER.

Random probing of plant expression libraries with MP as bait in two-hybrid or Far-Western screenings appears promising in the identification of host factors that show MP affinity. However, candidate host proteins may be involved in different distinguishable steps of the entire movement process. In this thesis research was focussed on the identification of plasma membrane-residing factors involved in CPMV movement.

OUTLINE OF THIS THESIS

Intensive studies over the past decade have provided a detailed insight into the mechanisms of cell-to-cell movement of plant viruses, in particular with respect to the viral factors involved. However, successful virus movement is a concerted effort of viral factors and host factors and our knowledge of the latter is very limited.

Considering the tubule-guided movement mechanism of CPMV, it is obvious that the MP must interact with components of the virions (the coat proteins) to achieve efficient encaging of these particles in the movement tubule. Furthermore, interactions with host factors for targeting to and anchoring at the plasma membrane are inevitable. The research reported in this thesis therefore focused on MP-interactions with CPMV virions and host (plasma membrane) components.

In **Chapter 2**, first the composition of the CPMV transport tubule was described, in terms of both viral and possibly host components. For this purpose a method to quantitatively purify membrane-bound transport tubules from infected protoplast suspensions had to be optimised. The purified tubules were then analysed for their protein composition to identify viral and host factors. In this study a deviant form of the small CP was found as one of the structural components of the virions encaged in the transport tubule. To address the question of whether this CP was directly involved in interactions between MP and virus particles, a prerequisite for the assembly of the virion-containing transport tubule, MP binding to virions and the separated CPs was studied *in vitro* using protein overlay assays and ELISA (**Chapter 3**). To provide MP probes for the binding assays, this protein first needed to be heterologously expressed and purified.

Having characterised MP binding to viral (structural proteins), in **Chapter 4** and **5** interactive activities of the MP with host factors were further investigated. In **Chapter 4**, the nature of a putative rNTP-binding domain in the CPMV MP was investigated, as

well as the potential of this protein to bind nucleic acids. In **Chapter 5,** the onset to identify MP-binding proteins in the plasma membrane of the host cell was described.

In **Chapter 6,** finally, the experimental results described in the previous chapters are discussed and integrated in a speculative model for the functioning of the CPMV MP during the viral cell-to-cell movement process.

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CHAPTER 2

Analysis of enriched membrane-bound movement tubule fractions from *Cowpea mosaic virus*-infected cowpea protoplasts

SUMMARY

To achieve cell-to-cell movement during the systemic infection process, progeny virions of *Cowpea mosaic virus* (CPMV) are translocated by means of a tubule assembled from viral movement protein (MP) copies inside the plasmodesmal pore. In analogy, virion-containing tubules enwrapped by plasma membrane are formed at the surface of infected host protoplasts. To study possible interactions between the viral MP and virus particles on one hand, and between the MP and host proteins on the other, these membrane-bound tubules were quantitatively isolated from infected protoplasts and analysed for their protein content. With respect to MP-virus binding it is shown that virus particles residing in the tubule contain a deviant S protein.

INTRODUCTION

As outlined in Chapter 1 *Cowpea mosaic virus* (CPMV) employs a movement mechanism involving the construction of a transport tubule within the cavity of structurally modified plasmodesmata to enable the translocation of mature virions across the cell wall. These transport tubules are composed of the viral movement protein (MP) and no other viral gene products are required for induction of the tubules (Wellink & Van Kammen, 1989; Van Lent *et al.,* 1990; Kasteel *et al.,* 1993; Wellink *et al.,* 1993; Kasteel *et al.,* 1996). Also the analysis of transport tubules, which were purified from infected cowpea protoplasts, did not reveal the presence of a (major) structural host specific protein (Kasteel *et al.,* 1997b) and thus it has been suggested that the viral MP is the sole structural component of the tubules.

In CPMV-infected plant cells, tubules are exclusively found inside the plasmodesma. In infected protoplasts a similar external polarity of the tubules is found and they are tightly surrounded by proliferated plasma membrane (PM) (Van Lent *et al.,* 1991). Tubules are formed after expression of the single MP gene in protoplasts isolated from both host and non-host plants, and even in animal (insect) cells (Kasteel *et al.,* 1996). These observations imply that the assembly of tubules inside plasmodesmata involves a specific interaction between the tubule (MP) and components residing in the PM. Such host components are not necessarily host- or even plant- (plasmodesma) specific, but the formation of tubules in insect cells suggests the involvement of factors which are conserved among plants and animals.

To approach the identification of PM-residing host components that may play a role in initiation of tubule assembly and in anchoring of the transport tubules, PM-bound tubules were isolated from CPMV–infected protoplasts and their protein composition was analysed.

MATERIALS AND METHODS

Virus and protoplasts

Cowpea mesophyll protoplasts were prepared from primary leaves of 10-day old cowpea plants (*Vigna unguiculata* ‘California Blackeye’), essentially as described by (Hibi *et al.,* 1975). All media contained 0.6 M mannitol and were adjusted to pH 5.7. One million protoplasts were inoculated with 10 µg purified CPMV SB strain using polyethylene glycol (PEG MW 6,000). Controls consisted of identical inoculations with UV-inactivated virus. Protoplasts were kept in sterile Erlenmeyer flasks and incubated in a growth chamber at 25°C and continuous illumination. At 40 h post-inoculation (p.i.) the percentage of infected protoplasts was determined by

immunofluorescent staining using an antiserum against the MP as described by Van Lent *et al.,* (1991).

Purification of tubules

Tubules were sheared from the protoplasts at 44 h p.i., by shaking the Erlenmeyer flasks on ice on a rotary shaker at 100 rpm for 20 min. Protoplasts were then pelleted by centrifugation for 5 minutes at 100 *g*. The supernatant containing the tubules was collected and placed on a 3 ml 25% (w/v) sucrose cushion in membrane-stabilizing buffer (MSB: 1 mM DTT, 0.1 mM EDTA, 3 mM $MgCl_2$, 10 mM KCl , 40 mM Hepes, pH 7.5) in a 35 ml Beckman SW28 centrifuge tube. The tubules were pelleted by centrifugation for 1 h at 53,000 g. The pellet was soaked and resuspended for 1 h in 0.5 ml MSB and centrifuged for 5 min at 1,000 rpm in an Eppendorf centrifuge to remove large aggregates. The tubules in the supernatant were subjected to equilibrium gradient centrifugation in 25% w/w caesium sulphate in MSB for 16 h at 145,000 g in a Beckman SW55 rotor at 10°C. The gradient was fractionated into 0.5 ml aliquots and fractions were analysed for their tubule content by negative staining electron microscopy (Van Lent *et al.,* 1991). Fractions were dialysed overnight at 4°C against three changes of MSB to remove the $CsSO_4$. Tubule-containing fractions were pooled and centrifuged for 5 min at 1,000 rpm in an Eppendorf centrifuge. Tubules were then concentrated by centrifugation for 1 h at 53,000 g, the pellet was resuspended in 100 µl MSB and the suspension was centrifuged for 5 min at 1,000 rpm to remove large aggregates. The supernatant containing the tubules was stored at 4°C until further analysis. Control suspensions were simultaneously prepared from protoplasts inoculated with UV-inactivated CPMV. The tubule suspensions were observed by negative staining electron microscopy.

PM fractions were prepared from cowpea leaves essentially as described by Larsson *et al.,* (1994).

Protein analysis

Purified tubule suspensions were analysed for protein content by SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 13.5 % w/v gels and gels were stained using a Silver Xpress (Invitrogen) silver staining kit according to the manufacturer's procedure. Viral proteins and selected host proteins were detected by immunoblotting. For this, SDS-PAGE gels were semi-dry blotted to immobilon P membrane and the membranes were immuno-probed with a mixture of rabbit antibodies against the MP (final dilution 1:3000) and anti-CPMV (final dilution 1:1500) or antibodies directed against host proteins. Primary antibody reaction was

detected using alkaline phosphatase or horseradish peroxidase conjugated to the appropriate secondary antibodies. Antibody binding was detected using the appropriate substrate for alkaline phosphatase or for horseradish peroxidase using the ECL Western blotting kit according to the Amersham protocol.

Antibodies

Polyclonal antibodies against CPMV were raised in rabbit against purified virus. Polyclonal antibodies against CPMV-MP were raised in rabbit against a peptide consisting of the 30 C-terminal amino acids of the CPMV MP (denoted anti-MP; (Wellink *et al.,* 1987)). Polyclonal rabbit serum against pectin methylesterase (PME) from *Nicotiana benthamiana* (Chen *et al.,* 2000) was kindly provided by Dr. M. H. Chen. Monoclonal mouse antibody against PME from tomato (Blumer *et al.,* 2000) was kindly provided by Dr. C.W. Bergmann. Polyclonal goat serum against clathrin from bovine brain was obtained from ICN Biomedicals (cat.no. 657811).

Polyclonal rat antibodies against AD3 protein from *N. benthamiana* were prepared as described by Van der Wel, (2000).

RESULTS

Purification of membrane-bound tubules

For each purification, portions of $5x10^7$ to 10^8 cowpea protoplasts were inoculated with CPMV and an equal number with UV-inactivated CPMV as a control. Invariably 80-90% of the protoplasts inoculated with CPMV became infected (Fig. 1A) and at 42 h p.i. approximately 60% of these infected protoplasts had tubule growth at the cell surface as was shown by immunofluorescent staining of the MP (Fig. 1B). The number of tubules induced per cell varied strongly from a few (1-5) to many (>20; Fig. 1B) and also their length was variable, the longest reaching up to 40 μm. Negative staining electron microscopy of whole mounts of infected protoplasts showed the PM to be tightly wrapped around the virion-containing tubular structure (Fig. 1C). The purification protocol summarised in Fig. 2 aimed at the separation of these tubules from the infected cell, the stabilization of the PM-bound tubular structure and the subsequent separation of the tubules from other host cell components.

Tubules were effectively sheared from the protoplasts by shaking, as immunofluorescent staining of the sheared protoplasts showed that most tubules had detached from the cell surface, while the protoplasts still retained their cellular integrity. The latter was important to allow easy and efficient separation of the tubule-containing incubation medium and host cell structures by low speed centrifugation.

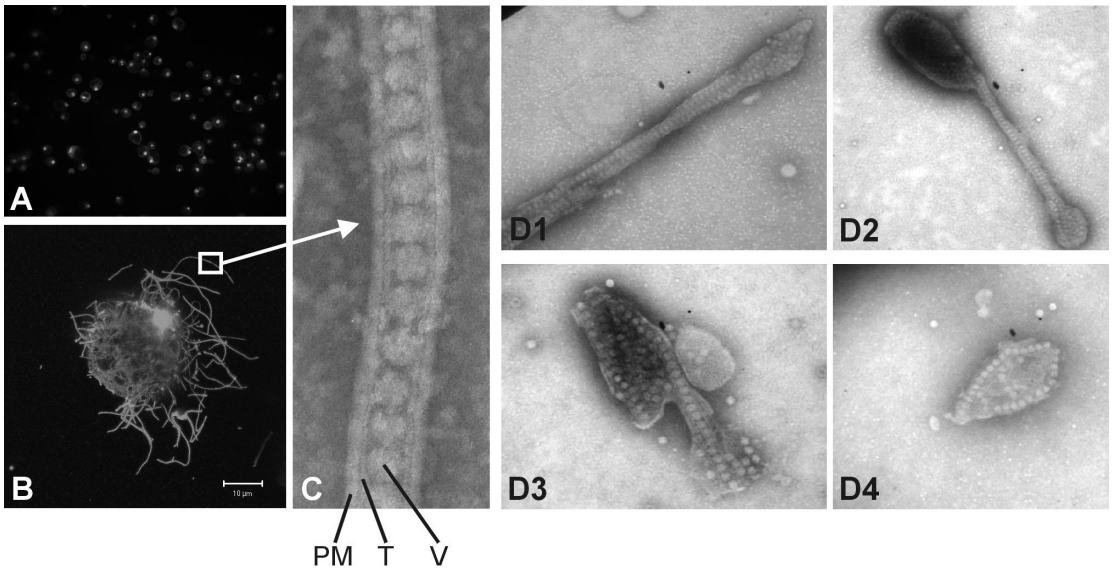

Figure 1 **(**A) immunofluorescent image of infected protoplasts showing nuclear staining of the viral MP and in more detail (B) showing numerous tubules at the cell surface. (C) negative stained EM image of a membrane-bound tubule in whole-mount preparations of protoplasts as depicted in B. D1-D4) Different forms of membrane-bound tubules observed in purified preparations obtained after density gradient centrifugation. PM= plasma membrane, T= tubule, V= virion.

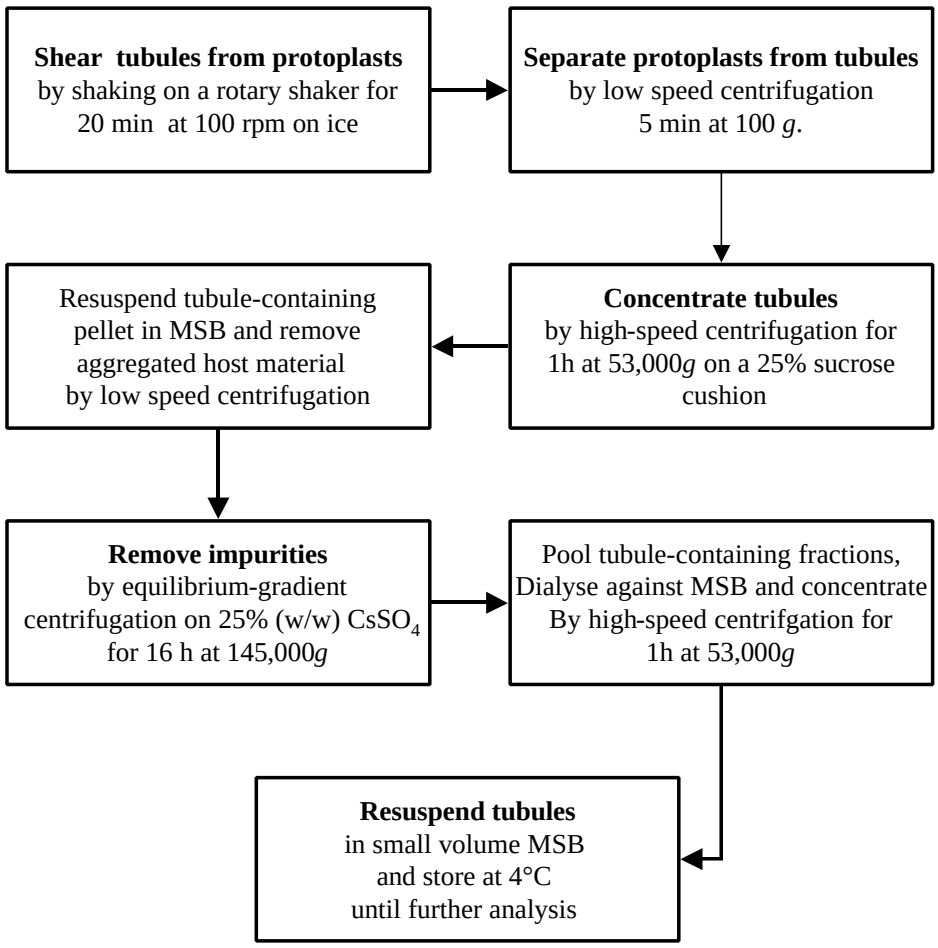

Figure 2 Diagram of the purification of membrane-bound tubules from protoplasts.

The subsequent steps in the procedure, the sucrose-cushion and $CsSO_4$ density gradient centrifugations, were executed to achieve further separation of the tubules from contaminating host constituents. The $CsSO_4$ gradients were fractionated into 10 aliquots of 0.5 ml and each fraction was analysed by negative staining electron microscopy for tubule content. Except for fractions 1 and 8-10, all fractions contained tubular structures still enwrapped by PM. The great variety in length and appearance of the PM-bound tubules (Fig. 1, panels D) apparently had resulted in widespread sedimentation of the structures over a large part of the density gradient. Fractions 2-7 were pooled, dialysed and concentrated by high-speed centrifugation. From each purification, the yield of tubules was evaluated by visual inspection of the sample by negative staining EM. Yields varied strongly between different purification attempts, but generally were very low.

Protoplasts inoculated with UV-inactivated virus showed no infection at 40h p.i. and were subjected to the same purification protocol to generate control samples. No tubules were ever observed in these samples.

Analysis of the protein content of purified tubules

The tubule-enriched samples prepared from CPMV-infected protoplasts, control samples and virus purified from the infected protoplasts at the time point of tubule isolation were analysed by SDS-PAGE and subsequent silver staining and immunoblotting using antibodies against CPMV virions and the MP (Fig. 3).

The silver stained gel and the immunoblots showed the presence of the viral MP and the two capsid proteins L and S in the purified tubule fraction but not in the control samples (Fig 3A and B). In the virus sample purified from the infected protoplasts, the L protein was detected, as were both expected electrophoretic forms of the S protein, denoted S-s (slow) and S-f (fast) (Taylor *et al.*, 1999). Remarkably, only a single form of this protein was found in the tubule samples (Fig. 3A and B indicated by the asterisk), while the estimated Mw of this protein was consistently larger (average 26.0 kDa from 4 different experiments) than the S-s form found in virus purified from the protoplasts (average 23.7 kDa from 6 different experiments). To exclude the possibility that the slow migration of S observed in the purified tubule fraction was caused by the composition of this sample (e.g. lipids), the tubule and virus fractions were mixed and analysed by PAGE and immunoblotting. All three forms of the S protein were resolved in the gel (Fig. 3D).

Besides these viral proteins, the silver-stained gels showed a large number of additional protein bands to be present in the tubule fraction when compared to the control samples. These proteins probably reside in the co-purified PM, which enwraps

the tubules. No prominent host protein band consistently re-appeared in 5 consecutively isolated tubule preparations (comparative data not shown).

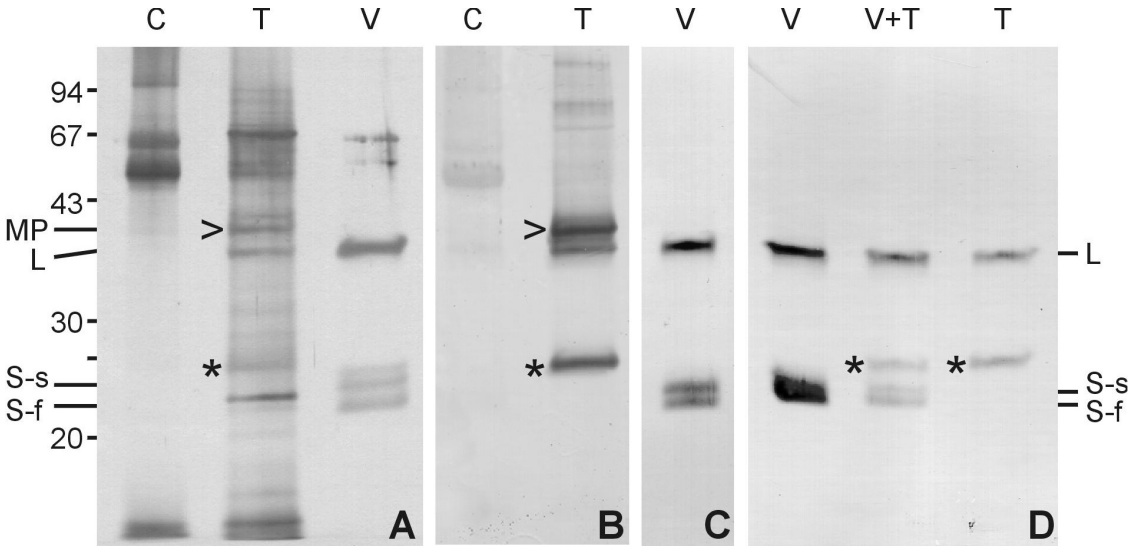

Figure 3 **(**A) Silver stained gel revealing the protein content of control samples (C) and samples containing membrane-bound tubules (T) and virus (V) purified from infected protoplasts. (B) and (C) Blots of similar gels probed with a mixture of antibodies against CPMV and MP (B), or antibodies against CPMV alone (C). (D) Blot of V, T and a mixture of both (V+T), probed with antibodies against CPMV. > indicates the MP; * indicates a slow migrating form of the S coat protein; L= large coat protein; S-s=slow migrating form of S protein; S-f= fast migrating form of S protein.

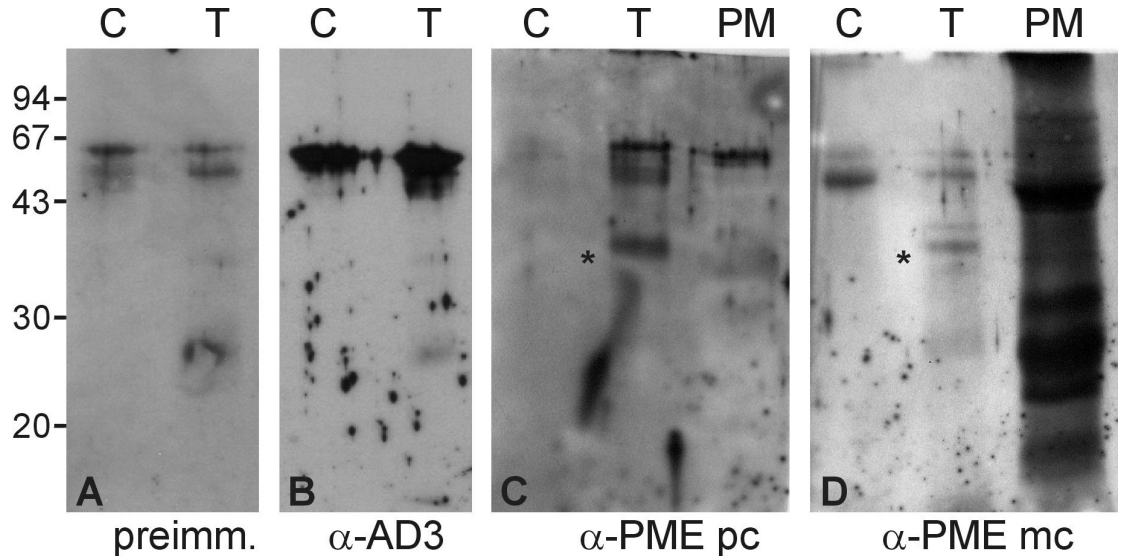

Figure 4 ECL blots of controls (C) and purified tubule fractions (T) probed with antibodies against different plant proteins implicated in virus movement. For further explanation see text. preimm.=pre-immune serum of AD3; pc=polyclonal; mc=monoclonal; *=PME. Positions of molecular mass markers are indicated on the left.

Testing purified tubule suspensions for host proteins

To date, only a few host proteins have been reported to associate with viral MPs and tentatively implicated in the mechanism of cell-to-cell movement of plant viruses. Pectin methylesterase (Chen *et al.,* 2000) and an unknown protein from *Nicotiana benthamiana* denoted AD3 (Chapter 6, (Van der Wel, 2000) have been shown to bind to tubule-forming viral MPs of some other viruses and to reside in the cell wall or PM. Antibodies against these proteins were used to detect their possible presence in membrane-bound tubules of CPMV. AD3 (Fig. 4B) could not be detected in the tubule fractions. However, a clear band (indicated by an asterisk) was observed with both a polyclonal (pc; Fig. 4C) and a monoclonal (mc; Fig. 4D) antibody preparation raised against PME. The mobility of the reactive protein (approx. 37 kDa) corresponded well with the size of PME (Chen *et al.,* 2000). Control samples and PM-enriched fractions purified from healthy cowpea leaves did not show a similar protein.

DISCUSSION

Analysis of the protein content of PM-delimited CPMV movement tubules shows the presence of the viral MP (as a constituent of the tubule), the two viral CPs (as constituents of the virions) and a large number of proteins that probably reside in or are attached to the tubule-enwrapping PM. It has previously been shown (Kasteel *et al.,* 1997a) that isolated CPMV tubules devoid of PM only contain detectable amounts of viral MP and CPs and consequently it has been suggested that these proteins were the only structural components of the tubule complex. Remarkably, the data shown in this chapter indicate that virions encased in movement tubules contain a version of the small coat protein (S CP), which migrates more slowly than S CP from virions isolated from the cytoplasm of infected cells. It is known that CPMV virions can be separated into two forms (f and s) according to their rate of migration toward the anode during electrophoresis (Agrawal, 1964). This difference in electrophoretic mobility is caused by a naturally occurring proteolysis of 24 amino acids from the carboxy-terminus of S (Taylor *et al.,* 1999). Virus preparations contain a higher proportion of processed S-f, when purified from plants in a late stage of infection (Niblett & Semancik, 1969; Geelen *et al.,* 1972). A deletion mutant lacking the C-terminal 24 amino acids of the S-CP and thus mimicking the naturally processed form, shows limited local spread and delayed systemic infection (Taylor *et al.,* 1999). These observations suggest an involvement of the C-terminal amino acids in targeting of the virions for intercellular movement. Our results show that in virus preparations isolated from protoplasts both described S forms are present, yet virions contained in transport tubules and produced

in the same cells show a unique form of S which migrates even more slowly than the unprocessed S-s form.

Given the precise mapping of the S-s form on the 3'-terminal end of the single open reading frame in RNA 2 it is not very likely that S from tubules has a longer amino acid chain. Hence, the altered electrophoretic mobility of S could point to a post-translational modification of the protein (e.g. glycosylation or phosphorylation). However, despite an earlier report (Partridge *et al.,* 1974) it has recently been unequivocally demonstrated (Altmann & Lomonossoff, 2000) that the CPs of CPMV are not glycosylated. It should be mentioned though that this study concerned virus particles that accumulated in the cytoplasm of infected cells. The molecular characteristics of the unique form of the S protein found in virions within transport tubules remain to be elucidated, but its presence may suggest a specific role for the S CP in cell-to-cell movement of the virions, e.g. in MP-virion interaction and/or sequestering and targeting of virions for intra- and intercellular transport. *In vitro* binding assays using the MP and the individual CP's could give clues to the importance of the S CP for cell-to-cell movement of CPMV. In Chapter 3 this binding is further analysed.

Electron microscopical analysis showed that the purified tubules were still encased by the PM. When comparing the content of the tubule fractions with that of fractions obtained from uninfected protoplasts, besides the aforementioned viral proteins (CP's and MP) numerous other proteins were present. These proteins most probably reside in or are associated with the encasing PM but their identity is hard to discern. A straight forward probing of the membrane-bound tubule fractions with antibodies against two proteins from *N. benthamiana* that are known to bind to tubule-forming MPs (PME and AD3) (Chen *et al.,* 2000; Van der Wel, 2000) showed the possible presence of PME but not AD3. PME, however, is not a membrane-residing protein but one localised in the cell wall. During incubation of the protoplasts some degree of cell wall regeneration may take place and cell wall material may therefore constitute a proportion of the isolated membrane-bound tubules. Members of the PME multigene family are involved in cell wall turnover and their activity is thought to modulate pH and ion balance and to affect cell wall rigidity (Micheli, 2001). Cell wall modulation could be a necessity for the modification of the plasmodesmal pore to enable the insertion of the viral transport tubule. With *Alfalfa mosaic virus* (AMV) in *N. benthamiana*, Van der Wel *et al.,* (1998) showed that the diameter of the plasmodesmata involved in virus transport transiently increased, probably to enable assembly of the transport tubule. A role for PME in such a process could be envisaged but remains speculative and a subject for further research.

To identify PM proteins that are possibly involved in MP anchoring, membrane-bound tubule fractions could be a suitable substrate for testing MP binding in blot-overlay assays. However, purification of tubules as done for the studies in this chapter, is laborious and yields are generally low. Therefore, in Chapter 5, PM proteins with affinity for the MP were identified by blot-overlay assays using PM fractions isolated from uninfected cowpea leaves and purified MP as overlay probe.

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CHAPTER 3

The C-terminal region of the movement protein of *Cowpea mosaic virus* is involved in binding to the large but not to the small coat protein.

SUMMARY

Cowpea mosaic virus (CPMV) moves from cell to cell as virus particles, which are translocated through a plasmodesmata-penetrating transport tubule made up by viral movement protein (MP) copies. To gain further insight in the roles of the viral MP and capsid proteins (CP) in virus movement, full-length and truncated forms of the MP were expressed in insect cells using the baculovirus expression system. Using ELISA and blot overlay assays, affinity purified MP was shown to bind specifically to intact CPMV virions and to the large CP, but not to the small CP. This binding was not observed with a C-terminal deletion mutant of the MP, although this mutant has retained the capacity to bind to other MP molecules and to form tubules. These results suggest that the C-terminal 48 amino acids comprise the virion-binding domain of the MP.

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INTRODUCTION

Cowpea mosaic virus (CPMV) is a plant virus that belongs to the genus *Comovirus* of the family *Comoviridae* (for review see Goldbach & Wellink, 1996 and Pouwels *et al.* 2002b). Cell-to-cell movement of CPMV is characterised by transport of mature virions through tubules that are assembled inside the plasmodesmal pore and which contain the RNA-2 coded 48 kDa movement protein (MP; Fig. 1) (Wellink & Van Kammen, 1989; Kasteel *et al.* 1993; Van Lent *et al.* 1990). Such a tubule-guided cell-to-cell transport system has been described for viruses of different genera including *Caulimovirus, Nepovirus, Bromovirus* and *Tospovirus* (Perbal *et al.* 1993; Wieczorek & Sanfacon, 1993; Storms *et al.* 1995; Kasteel *et al.* 1997). Similar tubular structures are formed at the surface of infected protoplasts and both in protoplasts and plant tissue, virions appear in a single and continuous row within the tubules (Van Lent *et al.* 1991). The morphology of the virion-containing tubule suggests that tubule-assembly from MP molecules and entrapment of the virions takes place simultaneously at or near the plasma membrane. Tubule assembly does not depend on the presence of virions or capsid proteins (CPs), as expression of MP alone in protoplasts also leads to the formation of (empty) tubules (Wellink *et al.* 1993).

In Chapter 2 of this thesis it was shown that virions present in the transport tubule contained a deviant form of the small (S) CP, suggesting a role for the S CP in cell-to-cell movement, possibly in MP binding. A specific affinity of the viral MP for the CPs or virions, probably is essential not only for the entrapment of virions during tubule assembly, but also for the targeted transport of virions from the cytoplasmic site of assembly to the plasmodesma. Indirect evidence for a specific interaction between the C-terminus of the CPMV MP and virions was presented by Lekkerkerker *et al.* (1996), who showed that a mutant virus coding for a MP lacking the C-terminal 18 amino acids (residues 313-331) was able to form tubules in protoplasts, which do not contain virus particles, and the mutant was not able to spread systemically in plant tissue. Furthermore, for *Grapevine fanleaf nepovirus* (GFLV), another member of the family *Comoviridae*, Belin *et al.* (1999) found that the nine C-terminal amino acids of the MP must be of the same virus origin as the CP for successful systemic spread, and they also proposed a requirement for specific interactions between the movement protein ($2B^{MP}$) and the coat protein ($2C^{CP}$).

We have here further investigated the affinity of the CPMV MP for virions and for the individual viral CPs by *in vitro* binding assays, i.e. ELISA and blot overlay assays, using purified MP as a probe.

MATERIALS AND METHODS

Heterologous expression of MP

The 48 kDa MP and two truncated forms of this protein with six histidine (HIS) molecules linked to their N-terminus were expressed in *Spodoptera frugiperda* (cell line Sf21) insect cells using the BAC-to-BAC system (Gibco BRL).

To isolate the complete MP-coding sequence plasmid *pM19GFP2A* (Gopinath *et al.* 2000), containing the full length cDNA of CPMV RNA-2, was used as starting material. The MP fragment was synthesised by PCR using the primers 5'GGGGTACCATGGAAAGCATTATGAGC3' and 5'GGGCTAAGCTTTAGGCCT ATTGTGGAAAAGC3'. A KpnI site was introduced at the 5' end directly before the start codon of the MP sequence and a Hind III site including a stop codon at the 3' end (indicated in boldface). To generate the ΔC48MP, the same forward primer was used and a stop codon and HindIII site were placed at aa position 298 using the primer 5'GGGCTAAGCTTCTAAGAAATAGAGTATTTCAA3'. The fragment coding for ΔN289MP was amplified using primers 5'GGGGTACCGGAGAAAGTTTGAAA TACTCT3' and the reverse primer that was used for the full-length construct.

The fragments were first cloned into the pGEM-Teasy vector (Promega), excised with Kpn I and Hind III and inserted downstream of the polyhedrin promoter and an N-terminal 6xHIS-tag of plasmid pFASTBAC-HT (Gibco BRL). The constructs were sequenced to confirm the integrity of the insert. The resulting plasmids were named pFASTBAC-MP, pFASTBAC-ΔN289MP and pFASTBAC-ΔC48MP. Recombinant viruses, expressing the MP or the two truncated variants, were constructed using the BAC-to-BAC system protocol (GIBCO BRL) and the viruses were designated AcNPV-MP, AcNPV-ΔN289MP and AcNPV-ΔC48MP.

Sf21 cells were infected with each recombinant virus and at 36 h or 48 h p.i. the cells were harvested and checked for expression of the proteins by Western blot analysis (Gopinath *et al.* 2000) and immunofluorescence (essentially as described by Kasteel *et al.* 1996) using goat anti-rabbit conjugated to Alexa 488 as fluorochrome (Molecular Probes). Fluorescence was recorded in a Zeiss LSM 510 laser scanning microscope through excitation with blue laser light at 488 nm and emission through a 505-530 nm bandpass filter.

Purification of MP

The HIS-tagged MP and ΔC48MP were purified from AcNPV-MP and AcNPV-ΔC48MP infected cells using a Talon^{TM} Cell Thru column (Clontech). Infected and uninfected insect cells were pelleted and resuspended in 5 ml lysis buffer (50 mM sodium phosphate, 6 M guanidine-HCL, 300 mM NaCl, pH 8.0) containing 30 μg/ml

E64 protease inhibitor (Roche). Cells were ruptured by pulse sonication for 6 x 30 sec with 30 sec intervals and then centrifuged at 1,200 rpm in a SS34 Sorvall rotor for 20 min to pellet cell debris. The supernatant was collected and loaded onto a 2 ml bed volume Talon^{TM} Cell Thru column, previously equilibrated with lysis buffer. The column was first washed with 30 ml lysis buffer followed by 5 ml washing buffer (45 mM sodium phosphate, 5.4 M guanidine-HCl, 270 mM NaCl, 10 mM imidazole, pH 8.0). The MP or ΔC48MP was then eluted under denaturing conditions, with 3 ml of washing buffer containing 200 mM imidazole. The eluted proteins were immediately renatured by a sequence of dialysis steps against renaturing buffer (20 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10% glycerol, 100 mM NaCl, 7 mM 2-mercaptoethanol) containing decreasing concentrations of guanidine-HCl. Starting with 6 M guanidine-HCl for 6 hours, the concentration was halved every 6 hours by adding an equal volume of cold renaturing buffer until 750 mM. Except for the first dialysis, which was done at room temperature (RT), all dialysis steps were done at 4°C. Samples were then dialysed against pure renaturing buffer to remove guanidine-HCl. Protein concentration was determined by Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard. Purified MP was stored in 50 µl aliquots of 0.1 - 0.2 mg/ml at –80°C until further use.

Antibodies

Three antibodies were available to detect the HIS-tagged CPMV MP. A monoclonal antibody against 6xHIS (anti-HIS) obtained from Clontech could be used to detect denatured forms but not renatured forms of the MP and ΔC48MP. A rabbit polyclonal antibody against a peptide consisting of the 30 C-terminal amino acids of the CPMV MP (denoted anti-48K; Wellink *et al.* 1987) was most specific to the viral MP, but was not suitable to detect the C-terminally truncated ΔC48MP. A rabbit polyclonal antibody (anti-58K) generated against *E. coli*-expressed 58 kDa protein from CPMV RNA-2, which contains the entire 48 kDa MP sequence (denoted anti-58K; Kasteel, 1999) was suitable to detect both the MP and ΔC48MP but generally showed some additional bands in Western blots. Insect cell lysates and purified MP and ΔC48MP protein fractions were resolved on a 12% SDS-PAGE gels (Laemmli, 1970), which were stained with Coomassie blue or electro-blotted (0.04 mA for 1 hour at RT) to PVDF Immobilon P membrane (Amersham) in transfer buffer (50 mM Tris-base, 192 mM glycine, 20% methanol, 0.01% SDS, pH 9.5). On the latter, the HIS-tagged MP and ΔC48MP were detected with an anti-6xHis monoclonal antibody (Clontech) or anti-MP polyclonal antibody.

ELISA-based binding assay

Purified MP and ΔC48MP were tested for binding to virions in an ELISA assay. Wells of Nunc axisorp F96 immunoplates were coated with 150 µl of 6.6 µg purified CPMV/ml coating buffer (0.05 M sodium carbonate, pH 9.6) overnight at 4 °C. Controls consisted of purified MP and similar amounts of purified virions of related comoviruses *Cowpea severe mosaic virus* (CPSMV) and *Red clover mottle virus* (RCMV) and the unrelated *Tobacco mosaic virus* (TMV). After rinsing the plates three times with phosphate buffered saline (PBS), pH 7.2, containing 0.05% (v/v) Tween-20 (PBS-Tween), the wells were blocked with PBS-Tween containing 5% (w/v) dry non-fat milk for 1 hour. The plates were then incubated with 150 µl of 13 µg/ml purified MP or ΔC48MP in PBS-Tween containing 1% dry non-fat milk or PBS-Tween as a control for 90 minutes at RT. The plates were rinsed three times with PBS-Tween and incubated with anti-48K or anti-58K antibodies at 37° C for 1 hour. The plates were again rinsed and incubated with secondary antibody conjugated to alkaline phosphatase at 37° C for 1 hour. After rinsing 150 µl substrate (1 mg p-nitrophenyl phosphate disodium/ml 0.01 M diethanolamine buffer, pH 9.6) was added per well. Reactions were quantified by reading the absorbance at 405 nm using a Bio-Tek Instruments EL 312 ELISA-reader. Samples giving an ELISA reading higher than the average of six control readings plus 3 times the standard deviation were considered positive, those with lower readings negative.

Blot overlay assay

Binding properties of the MP and ΔC48MP were further explored using blot overlay assays essentially as described by Chen *et al.* (2000). Ten µg of purified MP or ΔC48MP and different virus suspensions (CPMV, RCMV, CPsMV and TMV) were resolved on a 12% SDS-PAGE gel and electro-blotted onto a PVDF Immobilon P membrane. The membrane was washed twice in denaturating buffer (6 M guanidine hydrochloride, 2 mM EDTA, 50 mM DTT, 50 mM Tris-HCl, pH 8.3) for 10 minutes at RT. The proteins were slowly renatured in serially diluted renaturating buffer (10 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 2 mM EDTA, 2 mM DTT and 0.1% (v/v) Nonidet P-40) containing resp. 4, 3, 2, 1 and 0 M guanidine-HCl each for 10 minutes at 4 °C. The renatured blot was blocked overnight in renaturating buffer containing 5% (w/v) non-fat dry milk. To test MP and ΔC48MP binding, the blot was incubated for 90 minutes at RT with 10 µg/ml of either protein in renaturating buffer containing 1% non-fat dry milk and 5% (v/v) glycerol. The membrane was washed four times for 5 min with washing buffer (TBS with 0.2% (v/v) Triton-X-100), incubated for 1 hour at RT with anti-48K or anti-58K antibody and washed again four times for five minutes

in washing buffer. The membrane was then incubated with the secondary antibody for 1 hour at RT and washed four times for five minutes with washing buffer. Antibody binding was detected using the ECL Western blotting kit according to the Amersham protocol. Alternatively, blots containing the MP were probed with purified CPMV and virion binding was detected using anti-CPMV antiserum in a similar procedure.

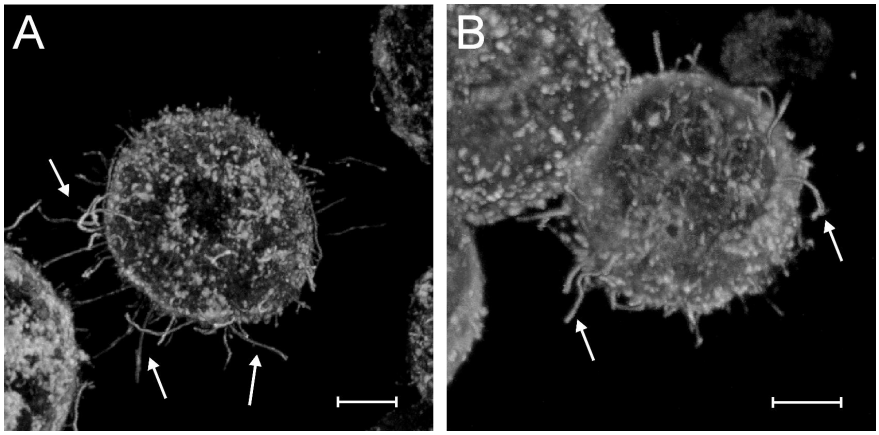

Figure 1 Immunofluorescent staining of wildtype MP (A) and ΔC48MP (B) in Sf21 insect cells 36 h p.i.. MPs were detected using anti-58K antibodies. Both proteins are capable of assembling into tubules (arrows) at the insect cell surface. Images are projections of series of two-dimensional scans. Bars represent 5 μm.

RESULTS

Expression and purification of MP

The recombinant baculoviruses AcNPV-MP, AcNPV-ΔN289MP and AcNPV-ΔC48MP, were used to express the MP and truncated forms of this protein in Sf21 insect cells. The truncated MP sequences were designed, on the basis of the domain analysis of Lekkerkerker *et al.* (1996) and Bertens *et al.* (2002), to generate a mutant MP with a deletion of 48 C-terminal amino acids (ΔC48MP) lacking the putative virion-binding domain and a peptide consisting of the 52 C-terminal amino acids (ΔN289MP) constituting the putative virion-binding domain. All proteins were expressed with six histidines linked to their N-terminus. The MP (AcNPV-MP) and the truncated MP with a deletion of the C-terminal 48 amino acids (AcNPV-ΔC48MP) were highly expressed and readily detected by immunofluorescence (Fig. 1) and on Western blots with cell lysates using the anti-58K antibodies (not shown). In cells infected with AcNPV-MP (Fig. 1A) and AcNPV-ΔC48MP (Fig 1B) the proteins were present in small and large aggregates mostly located in the periphery of the cell near the plasma membrane. Furthermore, both the MP and the truncated ΔC48MP formed tubules extending from the insect cell surface into the culture medium, indicating that

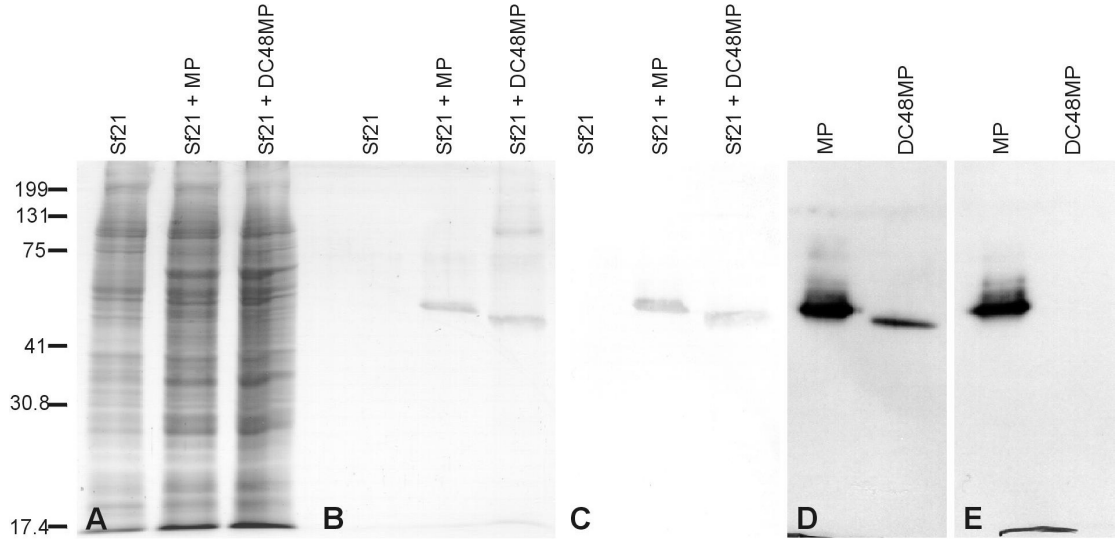

Figure 2 Coomassie blue stained gel of (A) lysates of uninfected (Sf21) and AcNPV-MP or -ΔC48MP infected insect cells and of (B) MP and ΔC48MP suspensions purified from these lysates. (C) Western blot of gel similar to B, treated with anti-HIS antibodies showing specific detection of MP and ΔC48MP. Lanes Sf21 in B and C contain fractions purified from uninfected Sf21 cells. D + E) Binding overlay assays of purified MP and ΔC48MP probed with MP (D) and buffer (E). Binding was detected using anti-48K antibodies. The strong signal in the MP lanes is the result of direct binding of the anti-48K antiserum to the blotted MP. Positions of molecular mass markers are indicated on the left.

the tubule-forming capacity is not disturbed by the N-terminal tag and the C-terminal deletion. In cells infected with the AcNPV-ΔN289MP no expression of the truncated peptide could be detected by immunofluorescence or Western blot assays using the anti-48K antibodies which are specific for the 30 C-terminal amino acids of the CPMV MP (Wellink *et al.* 1987). The peptide was either not expressed or expressed in amounts that could not be detected.

The HIS-tagged MP and ΔC48MP were purified from insect cell lysates using Talon column filtration. Initial attempts to purify the proteins from the cell lysates in native condition failed. Probably the proteins were aggregated to multimeric forms and/or the HIS-tag was not sufficiently exposed for binding to the column. Both proteins were successfully purified under denaturating conditions after addition of 6 M guanidine-HCl to the lysis buffer prior to loading on the column. Fig. 2 shows a Coomassie blue stained gel of insect cell lysates (A), purified MP and ΔC48MP (B) and a Western blot of these purified proteins detected with anti-58K antibodies (C). After column purification, the eluted proteins were slowly renatured for further use in binding assays. From $5x10^7$ infected insect cells usually 170 - 210 μg of the MP could be obtained.

Binding between MP molecules

The availability of an antiserum specific for the C-terminus of the MP and a C-terminal deletion mutant of the MP allowed us to test the binding between MP molecules *in vitro* using a blot overlay assay. After renaturation, a membrane containing MP and ΔC48MP was incubated with MP. Binding between MP and ΔC48MP was detected using anti-48K antibodies, which only react to the MP but not to the C-terminally truncated ΔC48MP (Fig. 2D). The strong signal in the MP lane of 2D and E is the result of direct binding of the anti-48K antiserum to the blotted MP, while Fig. 2E demonstrates that this antibody does not react to the ΔC48MP.

Binding of MP to virions

Binding of the MPs to virions of several different viruses was tested in an ELISA assay. The wells were coated with purified virus suspensions or buffer and incubated with MP or ΔC48MP and bound MP was detected using anti-58K antibodies. Average results of three repetitions are illustrated in Fig. 3. The MP showed binding only to homologous CPMV virions, but not to virions of the related comoviruses RCMV and CPSMV or to virions of the unrelated TMV. The truncated ΔC48MP did not show binding even to the homologous CPMV virions.

Binding of the MP to virions could also be shown in a blot overlay assay, where the MP and ΔC48MP were blotted on the membrane and purified CPMV virions were used as the overlay probe. In this assay, virions bound to the MP but not to ΔC48MP (Fig. 4D).

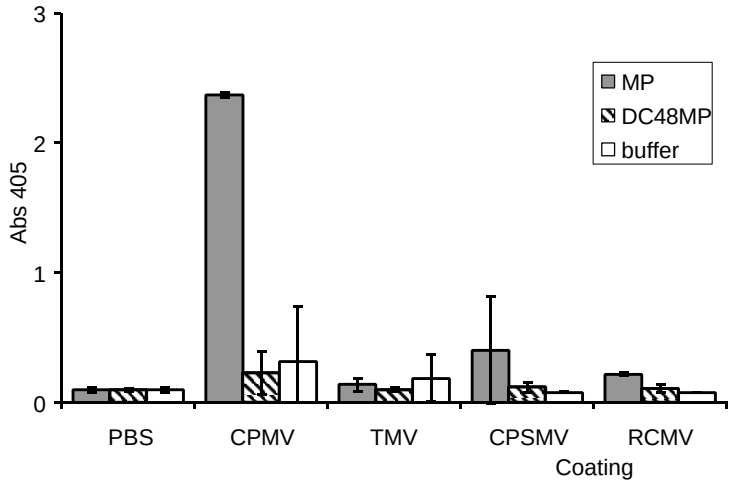

Figure 3 ELISA-based binding assay showing specific binding of MP, but not the ΔC48MP, to CPMV virions. Absorbance values represent averages of three consecutive experiments. Neither protein shows significant binding to virions of the related comoviruses CPSMV and RCMV or to TMV.

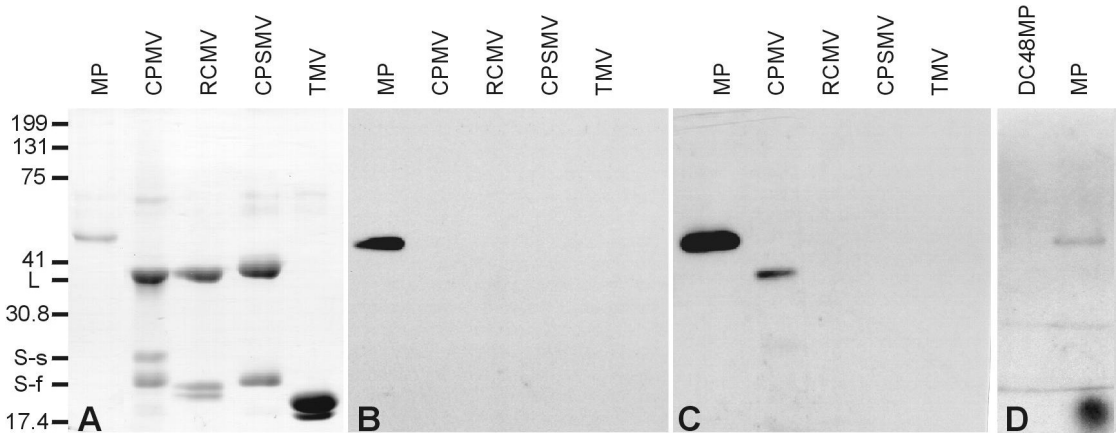

Figure 4 Coomassie blue stained gel (A) showing the purified MP and the CPs of purified virions of CPMV, RCMV, CPSMV and TMV. (B) and (C) show blot overlay assays probed with buffer and purified MP respectively and anti-58K antiserum. Binding of the MP only occurs with the L protein of CPMV, but not with the CP of other viruses. (D) Blot overlay assay of MP and ΔC48MP probed with CPMV virions and anti-CPMV serum. CPMV virions bind to the MP but not to the truncated ΔC48MP. The strong signal in the MP lanes is the result of direct binding of the anti-58K antiserum to the blotted MP. L = large CP; S-s = small CP slow running component; S-f = small CP fast running component.

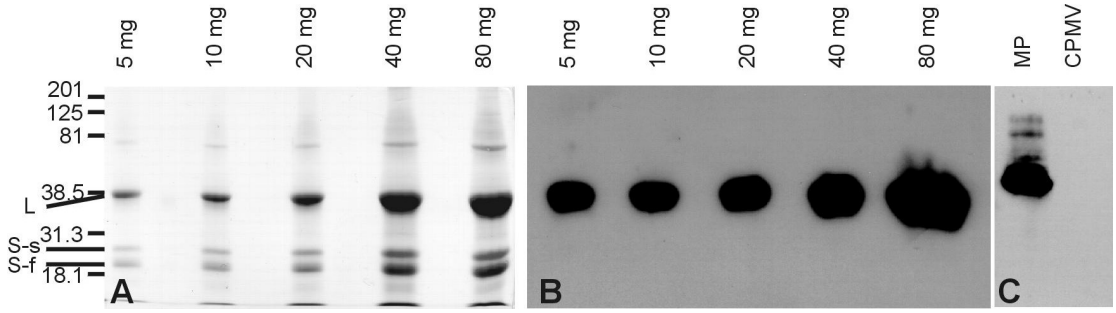

Figure 5 Coomassie blue stained gel (A) showing the separated L and S proteins of purified CPMV in increasing concentrations (5 – 80 μg). (B) shows an overlay assay of a blot from a similar gel probed with purified MP and anti-48K antibodies. MP only binds to the L protein but not to the S protein. (C) Overlay assay of a blot with purified MP and CPMV (10 μg virus) probed with the truncated ΔC48MP and anti-58K antibodies. The truncated MP does not bind even to the L protein. The strong signal in the MP lane is the result of direct binding of the anti-58K antiserum to the blotted MP. L = large CP; S-s = small CP slow running component; S-f = small CP fast running component. Positions of molecular mass markers are indicated on the left.

Binding of MP to separate CPMV coat proteins

As the MP specifically interacted with CPMV virions, it was interesting to investigate whether the protein binds to both L and S CP. Binding of the MP was tested in blot overlay assays resolving both CPs from increasing amounts (5, 10, 20, 40 and 80 μg) of purified CPMV. Bound MP was detected using anti-48K antibodies. Probing of the blotted membrane with MP revealed specific binding to the L protein, but not to the S protein of CPMV even at higher amounts of blotted CPs (Fig. 5B). The truncated ΔC48MP, detected with anti-58K antibodies, showed no binding to either CP

(Fig. 5C; the strong signal in the MP lane is the result of direct binding of the anti-58K antiserum to the blotted MP). Like in ELISA, no binding of MP occurred to CPs of the other, related and unrelated, viruses (Fig. 4C).

DISCUSSION

The mechanism of tubule-guided virion movement, employed by CPMV to achieve cell-to-cell movement, implies a specific interaction between the transport tubule, which is built-up from the MP within the plasmodesmal pore, and mature virions. The data reported here indicate that purified and solubilised CPMV-MP molecules bind to mature virus particles in *in vitro* ELISA-based binding assays and conversely, virions bind to the MP in western blot overlay assays. It was further shown that upon removal of 48 amino acids from the C-terminus of the MP, virion binding did not occur any more in either assay. As in both MP and ΔC48MP the tubule-forming domain appears to be functional, as indicated by the formation of tubules at the insect cell surface (Fig. 1), these results suggest that the C-terminal 48 amino acids are essential for the interaction between MP and virions and therefore probably comprise the virion-binding domain of the MP or an essential part of this domain. As it has been shown previously that C-terminal deletions in the MP sequence result in virus mutants producing empty MP tubules and deficient to move from cell-to-cell (Lekkerkerker *et al.* 1996; Bertens *et al.* 2003), the *in vitro* binding studies reported here reveal a functional binding between MP and virions, in particular the L protein.

As reported in Chapter 2 of this thesis, virions encased in transport tubules contain a deviant form of the S protein suggesting a role for this CP in virus movement. It was speculated that this CP could be involved in MP binding, but the binding assays presented here do not show any affinity of the MP for this CP. The role of the S CP in the viral movement process therefore remains enigmatic.

Heterologous expression of viral MPs in insect cells and subsequent column purification appears to be a suitable method to obtain functional probes for *in vitro* binding studies. Earlier studies have shown that upon expression of CPMV MP, but also MPs of other viruses employing a similar mechanism of tubule-guided virion movement, the MP retains its tubule-forming capacity in insect cells (Kasteel *et al.* 1996; Storms *et al.* 1995). Here it was shown that also a truncated form of the MP, containing the tubule-forming domain but devoid of the virion-binding domain, had retained its biological function. The MPs are located at the plasma membrane and assemble into tubules protruding from the cell surface into the culture medium, similar to that of MP in virus-infected plant cells (Van Lent *et al.* 1991; Bertens *et al.* 2003). Attempts to express a peptide of 52 C-terminal amino acids, comprising the putative

virion-binding domain, were not successful. The peptide was either not expressed or synthesised in amounts below the detection limit.

Purification of the expressed MPs was only feasible after unfolding of the proteins in the insect cell lysate by a procedure of denaturing prior to column filtration. Denaturing apparently exposed the HIS-tagged N-terminus sufficiently for efficient binding to the Talon column. Prior to their use as probes in binding assays, the MPs were renatured again. The process of denaturing and renaturing apparently did not affect the intermolecular binding of the MPs (essential for tubule assembly), as in blot overlay assays the MP probe showed binding to the blotted truncated ΔC48MP (Fig. 2D and E).

A remarkable outcome of the binding assays is the specificity of the MP for its homologous virion. No binding occurred even to virions of related comoviruses RCMV and CPSMV (for similarities between comoviruses see Haudenshield & Palukaitis, 1998). These results suggest that *in vivo* only homologous virions can be moved from cell-to-cell by the viral transport tubule and that the MP is not able to assist the movement of virions of even related viruses. Little, but supportive evidence is available for this hypothesis from a study performed by Belin *et al.* (1999) on the nepovirus GFLV, showing that nine C-terminal residues of the 2B movement protein must be of the same virus origin as the 2C coat protein for successful systemic spread.

In western blot overlay assays, binding of the MP only occurs to the CPMV L protein but not to the S protein (Fig. 5). The significance of this binding for the mechanism of virion movement remains to be elucidated, but the capacity of the MP to bind *in vitro* to L protein molecules may also indicate a possible early interaction between the MP and this coat protein, i.e. this may occur already in the membranous cytopathic structure where CPMV replication, viral protein synthesis and virion assembly takes place (De Zoeten *et al.* 1974; Carette *et al.* 2000 and 2002). MP may thus also be involved in the targeting of virions to the plasmodesma. It is still obscure how the virion-containing transport tubules are assembled, i.e. how the MP and virions are targeted to the plasmamembrane/plasmodesma. Recently, Pouwels *et al.* (2002a) showed that neither the cytoskeleton nor the secretory pathway play an essential role in this process.

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CHAPTER 4

The movement protein of *Cowpea mosaic virus* binds GTP and single stranded nucleic acid *in vitro*.

SUMMARY

To achieve cell-to-cell movement of virions, the movement protein (MP) of *Cowpea mosaic virus* (CPMV) forms tubules through plasmodesmata. In this study the MP has been analyzed for its potential to bind rNTP and nucleic acid. It is shown that purified CPMV MP is capable of binding rGTP but no other rNTPs. By site-directed mutagenesis the GTP binding site was located within the D-motif, a sequence motif conserved among the MPs of tobamo- and comoviruses. As the non-GTP-binding mutant MP was disturbed in intracellular targeting and tubule formation, it is concluded that GTP binding may play a significant role in targeted transport and, in analogy to tubulin, multimerization of the comoviral MP. Furthermore, it is shown that CPMV MP is capable of binding both single stranded RNA and DNA, but not double stranded nucleic acids, in a sequence non-specific manner.

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INTRODUCTION

Most plant virus genomes code for one or more movement proteins (MPs), which are required for viral cell-to-cell movement. Based on their primary structure, MPs can be divided into several super families, one of which is the '30K' superfamily, related to the *Tobacco mosaic virus* (TMV) MP (Melcher, 2000a). Within this '30K' superfamily two basic mechanisms for cell-to-cell movement have been proposed (Lazarowitz & Beachy, 1999). TMV MP typifies one mechanism, where the MP modifies plasmodesmata allowing viral RNA-MP complexes to move from cell-to-cell. The other type of movement is tubule-guided movement of mature virus particles through drastically modified plasmodesmata.

Cowpea mosaic virus (CPMV), the type member of the *Comoviridae*, is one of the best-studied viruses that use tubule-guided movement. Mutational analysis of the MP showed that the N-terminus and central region of the MP are involved in tubule formation (Bertens *et al.*, 2000; Lekkerkerker *et al.*, 1996), while the C-terminus is required for interaction with the virus particles to be transported (Lekkerkerker *et al.*, 1996; Bertens *et al.*, 2000; Carvalho *et al.*, 2003,). Secondary structure comparisons of MPs belonging to the '30K' superfamily predicted a common central core (Melcher, 2000a). In this core a single amino acid, an aspartic acid, referred to as the D-motif, is almost absolutely conserved (Koonin *et al.*, 1991; Mushegian & Koonin, 1993), but its function has so far remained unresolved. MPs of como- and tobamoviruses have been suggested to contain an rNTP-binding motif (Chen & Bruening, 1992, Saito *et al.*, 1988). Furthermore, some MPs of the '30K' superfamily (for example TMV MP) have been shown to bind GTP (Li & Palukaitis, 1996). However, in no case has the GTP binding site been identified and no function yet been described, although it was suggested that GTP hydrolysis provides the energy needed for cell-to-cell transport (Li & Palukaitis, 1996).

The aim of the study presented in this Chapter was to elucidate whether CPMV MP is able to bind GTP and, if so, to determine the function of the GTP-binding. Furthermore, it has here been investigated whether the MP would be able to bind nucleic acid. The data obtained demonstrate that CPMV MP is able to bind both GTP and single stranded nucleic acids, but that the binding domains involved do not overlap.

MATERIALS AND METHODS

Expression and purification of MP

Wild type (wt)MP and mutant ΔC48MP, which has a deletion of 48 amino acids in the C-terminus of the MP, were expressed using the Bac-to-Bac system (Gibco BRL) and purified by Talon column filtration as described previously (Carvalho *et al.,* 2003). To express mutant AM5MP, in which amino acids V142 and D143 were replaced with alanines, the NdeI/BamHI fragment from pTMAM5, which contains a full-length cDNA clone of CPMV RNA2 with the AM5 mutation (Bertens *et al.,* 2000), and the NcoI/NdeI fragment from pFASTBAC-MP were ligated into NcoI/BamHI digested pFASTBAC-MP using a triple ligation. AM5MP was then expressed in insect cells and purified and immunolocalized as described by Carvalho *et al.* (2003).

rNTP binding

To establish rNTP binding, GTP-coupled agarose beads (Sigma) were used. These were washed three times with binding buffer (10 mM Tris-HCl, pH 7.8; 100 mM NaCl; 5 mM $MgCl_2$; 1 mM EDTA; 1 mM DTT; 0.1 mM PMSF; 10% glycerol). Five µg of purified wtMP or AM5MP were incubated with 100 µl binding buffer for 30 min at 4°C. Then 50 µl of pre-washed GTP-coupled agarose beads were added to the MP solutions and incubated for 60 minutes at 4 °C. Unbound proteins were then separated from the GTP-agarose beads by centrifugation at 14,000 rpm for 30 sec in an Eppendorf centrifuge. The beads were washed three times with binding buffer to remove any remaining unbound protein, and immobilized proteins were eluted from the beads by boiling in sample buffer (Laemmli, 1970). Proteins were analyzed on 12% SDS-polyacrylamide gels, electro-blotted onto a PVDF Immobilon P membranes and the MP was detected using an antiserum against the MP (Kasteel *et al.,* 1996b). The ability of the MP to bind rNTPs other then GTP was established in a competition experiment. For this 2.5 mM of the competitor rNTPs (GTP, ATP, CTP or UTP) were added to the MP solutions and incubated for 60 minutes at 4 °C. Subsequently, binding of the MP to GTP-agarose beads was tested as described before.

GTPase activity

One µl of [γ-^{32}P]-GTP was incubated with 1 µg of wtMP or AM5MP and binding buffer in a total volume of 50 µl and incubated for 2 hours at 25°C. For the positive control the soluble (S30) fraction of a cowpea leaf was used and water was used as a negative control. Hydrolysis of GTP was tested by thin-layer chromatography. The

mixtures were applied directly onto thin layer chromatography plates and the GTP and P were separated using 0.3 M sodium phosphate, pH 3.8, containing 1 M LiCl as solvent. Radioactivity was detected with a Storm 840 phospho-imager using the Image Quant program (Molecular Dynamics).

Transfection and analysis of protoplasts

For the construction of pMON-wtMP-YFP, the MP gene was amplified from pMON-MP using specific primers, thereby introducing a BglII site and a NcoI site. This fragment was digested with BglII and NcoI and cloned into BglII/NcoI digested pMON-YFP. PMON-AM5MP-YFP was made by ligation of the BglII/XhoI fragment from pTMAM5, containing the mutation, into BglII/XhoI digested pMON-wtMP-YFP. Cowpea mesophyl protoplasts were isolated and transfected with pMON-wtMP-YFP and pMON-AM5MP-YFP by polyethylene glycol as described by Van Bokhoven *et al*. (1993b). The protoplasts were cultured in continuous light at 25°C for 24 hours. Images from the protoplasts were taken with a Zeiss LSM510 confocal microscope using standard YFP filters.

Preparation of nucleic acids

To prepare radioactive ssRNA, the plasmid M19GFP7 (Gopinath *et al.,* 2000), which contains a full-length cDNA clone of CPMV RNA2 in which the GFP gene is introduced flanked by two artificial cleavage sites downstream from a T7 promoter, was linearized with PvuII resulting in transcription of the 3'terminal 707 nt of CPMV RNA2. *In vitro* transcription was carried out using T7 RNA polymerase (Gibco BRL) in the presence of [γ-^{32}P] CTP. Each reaction contained 1 μg of template DNA, 20 units of Rnase inhibitor (Rnasin, Gibco BRL), 1.25 mM of each rNTP (Promega), 25 units of T7 RNA polymerase and its buffer at appropriate final concentration as suggested by the manufacturer. The reactions were incubated at 37°C for 1.5 h and after the transcription the DNA templates were removed from the labelled RNA probes with a DNA-free RNA kit (Zymo Research).

For the competition experiments ssRNA and dsRNA competitors were used corresponding to the 207 3'-terminal nt of BMV RNA3 as described previously (Jansen *et al.,* 1998). For competition with ssDNA and dsDNA, M13 DNA and KpnI/HindIII digested plasmid pFASTBAC-HT (Gibco BRL) were used respectively.

Gel mobility shift assay

Different concentrations of purified and re-folded CPMV MP (0-1500 ng) were incubated with 50 ng radioactive CPMV RNA2 probe in 20 µl of binding buffer for 60 min at room temperature. For the competition assays the MP was first incubated with 1 µg of the competitor (20-fold excess) for 30 min, and then incubated for 60 min with 50 ng radioactive CPMV RNA2. After incubation, 6 µl of the mixture was used for electrophoresis in 1% agarose gels in TBE buffer. The gels were dried and autoradiographed.

RESULTS

CPMV MP binds GTP

To investigate potential rNTP-binding properties of CPMV MP, GTP-coupled agarose beads were incubated with purified wtMP, pelleted by centrifugation and washed three times to remove unbound proteins. Pellet (bound protein) and supernatant (unbound protein) fractions were analysed by immunoblotting using anti-MP antibodies. The immunoblot experiments (Fig. 1A) show that MP binds to the GTP-coupled beads. As a control and to confirm that the MP bound to the GTP and not the beads, the MP was incubated with 2.5 mM GTP prior to incubation with the GTP-agarose beads. In this competition experiment most of the MP remained in the

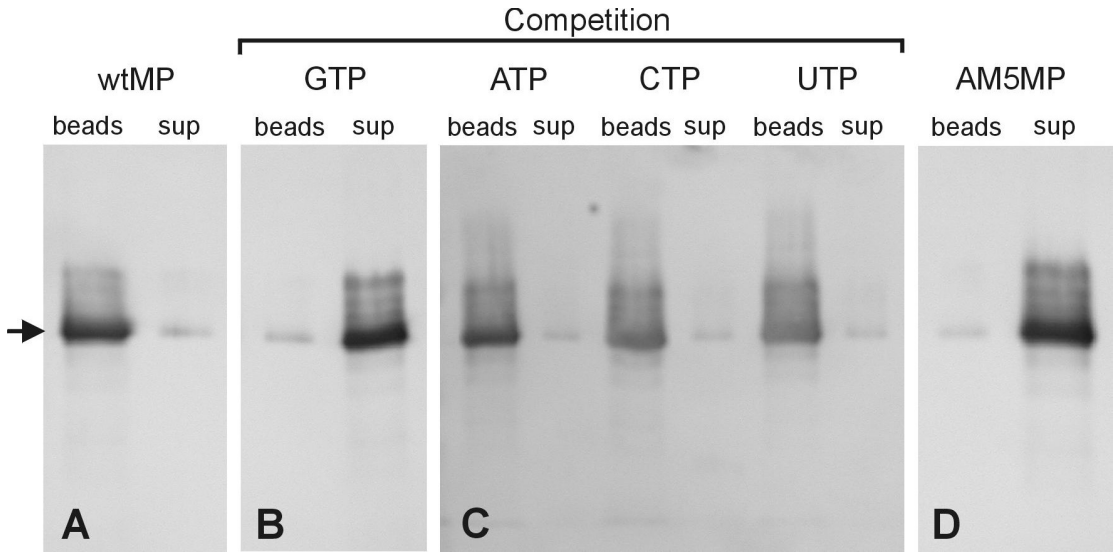

Figure 1 GTP-binding capacity of the CPMV MP. Purified MP was incubated with GTP-agarose beads (A). Unbound proteins (sup) were separated from GTP-agarose beads by centrifugation (beads), and both were analyzed by western blotting using anti-CPMV MP. For the competition assays the wtMP was incubated with 2.5 mM of GTP (B), ATP, CTP or UTP (C). Mutant AM5MP does not bind GTP. Arrow indicates the position of the MP (D).

supernatant fraction, indicating that the binding of MP is to the GTP and not directly to the agarose beads (Fig. 1B). To establish whether CPMV MP also binds other rNTPs, the protein was incubated with ATP, CTP or UTP prior to incubation with the GTP-coupled beads. In all three cases the MP still bound to the GTP-agarose beads and was present in the pellet fraction (Fig. 1C), indicating that the CPMV MP specifically binds GTP and not to other rNTPs.

In MP mutant AM5 (Bertens *et al.*, 2000), amino acids V142 and D143, which are located in the putative rNTP binding site (Chen & Bruening, 1992b), were substituted for alanines. Mutant AM5MP tagged with six histidines at the N-terminus was expressed in insect cells and purified from cell lysates using Talon column filtration (as described for wtMP by Carvalho *et al.*, 2003). The AM5MP was incubated with GTP-coupled agarose beads, and binding to GTP was investigated in the same way as the wtMP, showing that the replacement of V142 and D143 with alanine residues strongly inhibited the binding of the MP to GTP (Fig. 1D).

GTP binding by MP might play a role in targeting of MP to the cell periphery

To determine whether the mutation in the rNTP-binding site has an effect on the subcellular localization of the MP, insect cells (*Spodoptera frugiperda; Sf21*) expressing mutant AM5MP were fixed for immunofluorescent detection of the protein. In these cells AM5MP (Fig. 2A and B) accumulated in aggregates in the cytoplasm, as wtMP (Fig. 2C). Rarely, in approximately 1% of the infected insect cells, relatively

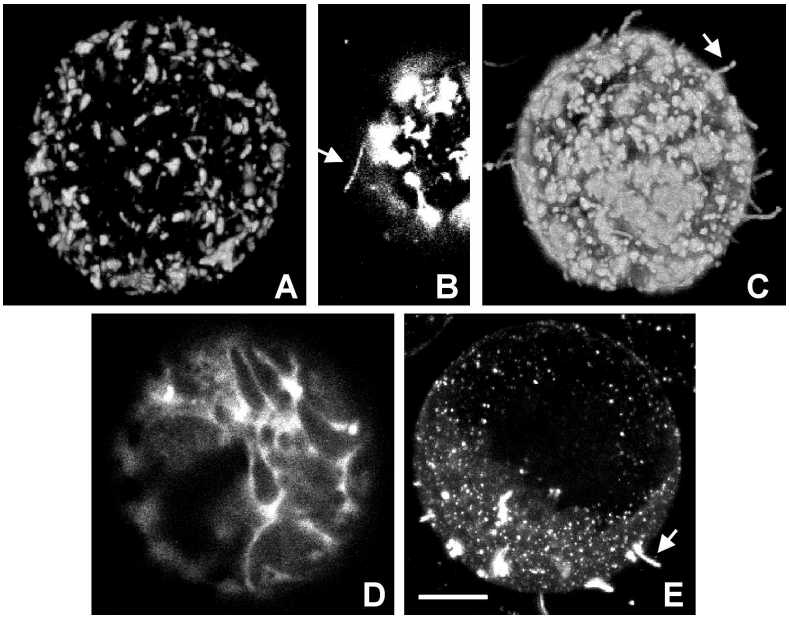

Figure 2 Intracellular localization of wt and AM5 mutant MPs in insect cells and cowpea protoplasts. (A-C) Immunofluorescent localization of MP in insect cells (*Sf*21) expressing AM5MP (A-B) and wtMP (C). (D) Cowpea protoplasts transiently expressing AM5MP-YFP and (E) wtMP-YFP. Arrows indicate tubules formed at the cell surface. Bar represents 5 μm.

short tubules were found at the cell surface (Fig. 2B), while tubules were present on approximately 80% of the infected insect cells expressing the wtMP (Fig. 2C), indicating that GTP-binding plays a role in tubule formation. Since wtMP formed aggregates in the cytoplasm, which is not observed in plant cells, and insect cells are not a host for CPMV, AM5MP fused C-terminally to YFP (AM5MP-YFP) was transiently expressed in cowpea protoplasts to confirm the localization pattern. At 24h post transfection, AM5MP-YFP had accumulated uniformly in the cytoplasm of transfected protoplasts (Fig. 2D), in contrast to wtMP-YFP, which accumulated in punctate structures at the plasma membrane and formed long tubules protruding from the cell surface into the culture medium (Fig. 2E), indicating a role for GTP binding in targeting MP to the plasma membrane.

CPMV MP has no intrinsic GTPase activity

As GTP-binding often coincides with GTPase activity of a protein, the capacity of CPMV wtMP and AM5MP to hydrolyze GTP was determined using [α-^{32}P]–GTP as a substrate. The positive control consisted of a cytoplasmic extract (the S30 fraction) of cowpea leaves and H_2O was the negative control. The results obtained demonstrate that neither wtMP nor AM5MP exhibit GTPase activity in this assay (Fig. 3), while GTPases present in the cytoplasmic fraction from cowpea leaves were able to hydrolyze GTP.

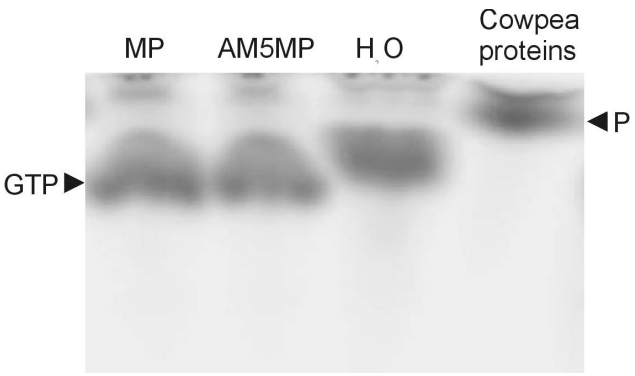

Figure 3 GTPase activity assay of CPMV MP. One µg of wtMP or AM5MP was incubated with [γ^{32}P]-GTP and applied onto thin-layer chromatography. A cytoplasmic extract of cowpea leaves was used as a positive control and ddH_2O as a negative control. The GTP and P are indicated.

CPMV MP is able to bind ssRNA and ssDNA in a sequence nonspecific manner

GTP binding by CPMV MP may be the result of RNA binding capacity. Although for many MPs of the '30K' superfamily RNA-binding has been confirmed, this information was not available for the CPMV MP. RNA binding of both wt and AM5

MPs was studied in a gel-shift assay using a radiolabelled 707 nt RNA fragment derived from CPMV. The gel shift assay shows that the wt protein is able to bind single stranded RNA in a cooperative manner (Fig. 4A). Heating for 5 min at 95°C prior to binding drastically decreased its RNA binding capacity (data not shown), indicating that the native protein conformation is important for RNA binding. RNA binding also decreased with increasing concentrations of NaCl (Fig. 4B) suggesting that binding involved ionic interactions. To determine the specificity of nucleic acid binding, competition experiments were performed with ssRNA, dsRNA (both corresponding to the 3'-terminal 203 nt of BMV RNA3 (Jansen *et al.,* 1998), ssDNA (M13 DNA), and dsDNA (pFASTBAC-HT digested with KpnI and HindIII). These analyses (data not shown) revealed that CPMV MP binds ssRNA or ssDNA without sequence specificity, but not dsRNA or dsDNA. RNA binding experiments were carried out with mutants AM5MP (Fig. 4C) and Δ48CMP (data not shown). In the latter mutant, the C-terminal 48 amino acids were deleted and this mutant was previously shown to be unable to bind CPMV virions or coat proteins (Carvalho *et al.,* 2003). Both mutant MPs had retained the RNA-binding capacity, indicating that different domains in the MP are involved in RNA binding, GTP binding and virion binding.

DISCUSSION

Comoviral and tobamoviral MPs have been suggested to contain Walker B-like rNTP binding sites (Saito *et al.,* 1988; Chen & Bruening, 1992b) and the MPs of TMV and *Cucumber mosaic virus*, which, like CPMV MP, belong to the '30K' superfamily of MPs, have been shown to bind GTP (Li & Palukaitis, 1996). Here, this property has also been shown for CPMV MP and moreover, we have mapped the binding site to be located around the D-motif as a mutant (AM5) with a modified D-motif was not able to bind GTP anymore. Although the lack of GTP binding of this mutant could be due to misfolding, this is very unlikely as the two amino acid changes in this mutant are rather mild and it still binds RNA, which we showed to be dependent on the conformation of the MP.

Within the D-motif, the aspartic acid probably plays a critical role in GTP binding, and based on the resemblance with the Walker B motif, it might be involved in binding an Mg-cation complexed with phosphate groups of the GTP (la Cour *et al.,* 1985; Fry *et al.,* 1986). The observation that the D-motif is highly conserved among members of the '30K' superfamily suggests that GTP binding may be a general feature of these MPs. So far a function for the GTP binding by MPs has not been demonstrated although it was suggested that rNTP binding and hydrolysis provide energy for cell-to-cell movement, which is supposed to be an energy-dependent

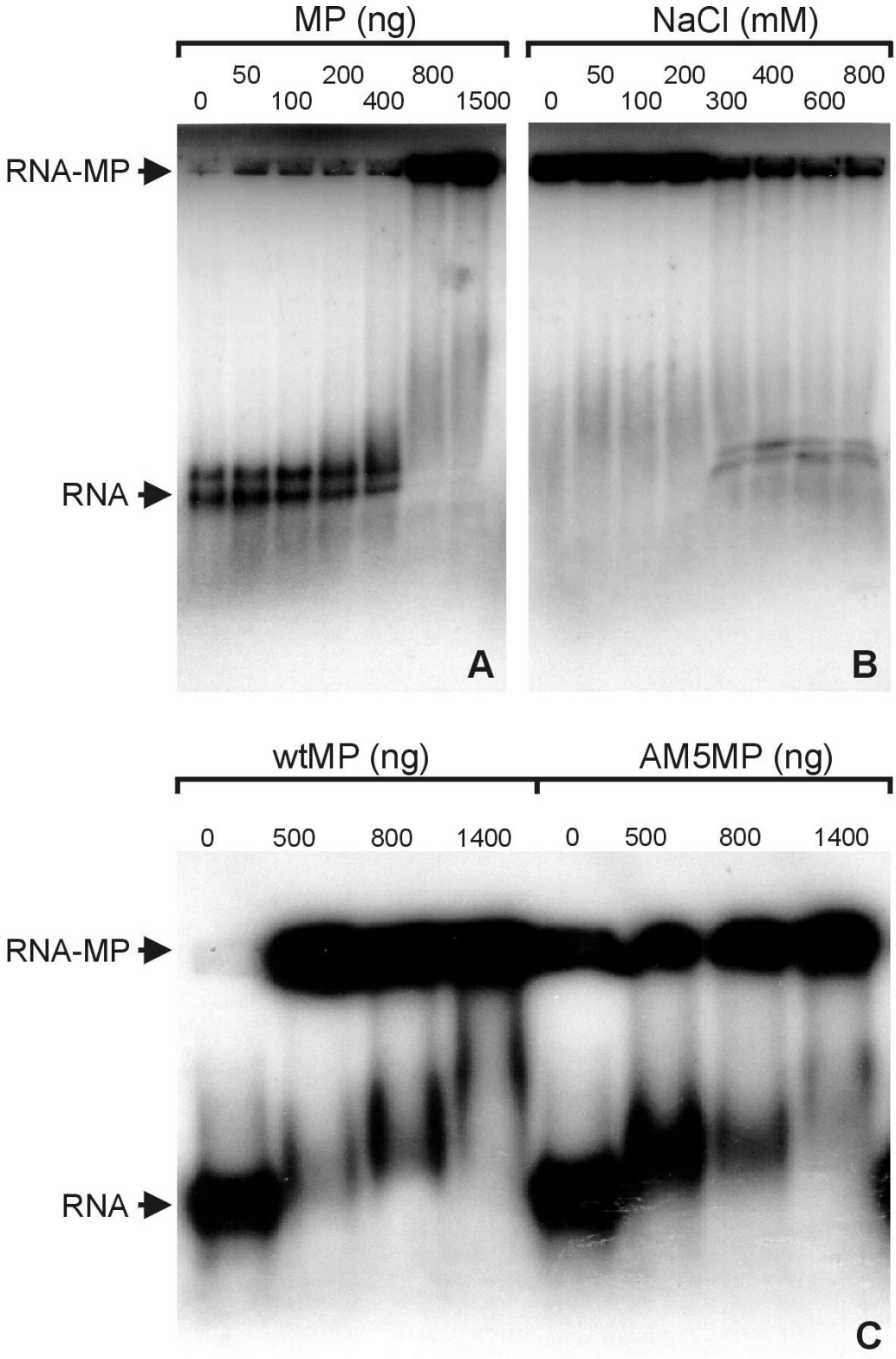

Figure 4 Nucleic acid binding of CPMV MP evaluated by gel shift assay. The protein-RNA complex and the free RNA were separated by electrophoresis in a 1% agarose gel. 50 ng of ^{32}P-labeled ssRNA corresponding to the C-terminal 707 nt of CPMV RNA2 was used in all experiments. For reasons yet unknown two RNA molecules were produced during *in vitro* transcription. (A) Binding of different amounts of MP (0~1500 ng) to ssRNA. (B) Binding of CPMV MP (500 ng) to ssRNA under increasing NaCl concentrations (0~800 mM). (C) Various amounts of AM5MP (0~1500) were incubated with 50 ng of 32P-labeled ssRNA. The wtMP was used as a control.

process (Carrington *et al.*, 1996; Ghoshroy *et al.*, 1997). At least two steps during MP-mediated cell-to-cell movement might involve such rNTP dependent events: (i) intracellular targeting of MP or MP-RNA complexes to plasmodesmata and (ii) modification of plasmodesmata either by trafficking of MP-RNA complexes or formation of tubules (Ghoshroy *et al.*, 1997; Heinlein, 2002; Lazarowitz & Beachy, 1999). The cytoplasmic location of mutant AM5MP in protoplasts (Fig. 2D) suggests a role for GTP binding in targeting MP to the plasma membrane, although in insect cells tubular structures could very rarely be observed (Fig. 2B). This might be caused by over-expression of MP in insect cells, which also would explain why both wt and AM5 proteins form aggregates in the cytoplasm of insect cells and not in protoplasts. The observations that the targeting deficiency of AM5 could not be complemented by wt protein (data not shown) and that targeting to the plasma membrane very likely requires di- or multimerization of MPs (Pouwels *et al.*, 2003) suggest that GTP binding might play a role in MP multimerization. It is tempting to speculate that tubules made by CPMV MP resemble microtubules since β-tubulin also requires GTP binding to be incorporated into microtubules (Howard & Hyman, 2003). Tubules made by CPMV MP probably disassemble in the neighbouring uninfected cells to release virus particles (Pouwels *et al.*, 2003). In analogy to microtubules (Hyman *et al.*, 1992), GTP hydrolysis could be required for this process. GTPase activity, however, could not be detected for CPMV MP when incubated with radioactive GTP (Fig. 3), although this experiment does not rule out that CPMV MP, like tubulin (Erickson & O'Brien, 1992; Nogales *et al.*, 1999), needs to form multimers or requires a host factor or membrane binding for GTP hydrolysis. For TMV MP, GTP binding (and hydrolysis) might be important for incorporation into microtubules, since this MP shows sequence similarity to a region in tubulins that mediate lateral contacts between microtubule protofilaments. This suggested that TMV MP might mimic tubulin assembly surfaces to propel viral RNA transport by a dynamic process that is driven by microtubule polymerization (Boyko *et al.*, 2000). As the D-motif plays a role in GTP binding by CPMV MP and is conserved throughout the '30K' MP superfamily, it is tempting to speculate that the D-motif is also involved in GTP binding by other MPs, where GTP binding may also be necessary for targeting to the cell periphery.

Gel-shift assays have indicated that CPMV MP, like the related *Broad bean wilt virus 2* (BBWV2) MP and many other MPs (Citovsky *et al.*, 1990; Citovsky *et al.*, 1991; Osman *et al.*, 1992; Schoumacher *et al.*, 1992; Li & Palukaitis, 1996; Qi *et al.*, 2002), is able to bind ssRNA and ssDNA in a sequence-unspecific manner but not dsRNA or dsDNA. The ability to bind single stranded nucleic acids therefore seems a general property of plant viral MPs. However, while the role of RNA binding for TMV MP is obvious since this MP needs to transport RNA, the significance of the RNA

binding capacity for CPMV MP has yet to be determined. In the case of CPMV, virus particles, which interact with the C-terminus of the MP in the tubules (Lekkerkerker *et al.,* 1996; Carvalho *et al.,* 2003), rather than MP-RNA complexes are transported from cell to cell. The formation of an MP-RNA complex, however, might be required for systemic infection. Alternatively, the capacity to bind RNA may not be important for the MP but may be required for the function of the overlapping CR protein, which is translated from a start codon in frame with that of the MP and plays a role in the replication of RNA2 (Van Bokhoven *et al.,* 1993a). However, we have not studied whether CR is able to bind RNA. A third possibility is that RNA binding is not needed at all for MPs that form tubular structures but is just a evolutionary remnant, although it seems unlikely that a protein of a plant RNA virus, which generally has a high mutation rate (Domingo & Holland, 1997), would retain such activity for no reason.

NaCl is able to disrupt the protein-RNA complex by interfering with the ionic interactions between the MP and RNA. The CPMV MP-RNA complex was disrupted at a NaCl concentration of 300 mM and higher, which is similar to values reported for *Alfalfa mosaic virus* MP (Schoumacher *et al.,* 1992) and *Cauliflower mosaic virus* MP (Citovsky *et al.,* 1991). The MP-RNA complexes made by TMV (Citovsky *et al.,* 1990), *Cucumber mosaic virus* (Li & Palukaitis, 1996), *Red clover necrotic mosaic virus* (Osman *et al.,* 1992), and BBWV2 (Qi *et al.,* 2002), however, dissociated at higher NaCl concentrations and therefore seem more stable. Whether the differences in the stability of the MP-RNA complex are biologically significant is unclear.

The observation that mutant AM5 is unable to bind GTP, but still binds RNA excludes the possibility that GTP binding by MP is due to binding of GTP to the RNA binding site. Previously, it has been shown that deletion of the D-motif, and consequently the putative rNTP-binding site, in TMV MP (Citovsky *et al.,* 1990) did not disrupt RNA binding, indicating that also for TMV MP GTP binding does not occur via the RNA binding site, although for TMV MP the GTP binding has not been mapped to the putative rNTP binding site yet.

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CHAPTER 5

Analysis of host plasma membrane proteins interacting with *Cowpea mosaic virus* movement protein

SUMMARY

During *Cowpea mosaic virus* (CPMV) infection the viral movement protein (MP) is targeted to the periphery of the cell where it assembles into tubules, which penetrate the plasmodesma, thus allowing the transport of progeny virions to the neighbouring cells. This mode of action implies an association between the viral MP and plasma membrane proteins of the host. To characterise these host proteins, purified CPMV MP was used as a probe in overlay assays and affinity column chromatography to assess plasma membrane fractions for the presence of MP-binding components. Using plasma membrane fractions from both cowpea and *Nicotiana benthamiana* in the overlay assays, MP-binding proteins with apparent sizes of 34, 30 and 28 kDa were detected. Further analysis of the cowpea plasma membrane fraction using affinity chromatography also revealed a limited number of MP-binding proteins including again a 30 kDa protein band. Sequencing of a tryptic digest revealed that the MP-binding 30 kDa protein band actually consisted of two protein species, an aquaporin and a vacuolar type ATPase. As both proteins are conserved among plants (and animals) and moreover are known to be localised in the plasma membrane, a specific role of these host proteins in viral MP functioning is suggested.

INTRODUCTION

To successfully infect host plants, viruses must spread from the initially infected cell through plasmodesmata to neighbouring cells, until they reach the phloem where they are transported systemically through the vasculature (for reviews see Carrington *et al.*, 1996; Lazarowitz & Beachy, 1999). Cell-to-cell movement is potentiated by viral movement proteins (MPs), which are targeted to the plasmodesmata and increase the size inclusion limit of these intercellular connections. Different viruses may utilize different mechanisms for cell-to-cell movement and plasmodesma modification. A major part of the plant viruses, exemplified by *Tobacco mosaic virus* (TMV) move their unencapsidated genome through plasmodesmata that are not grossly modified by their MP. Another category of plant viruses, exemplified by *Cowpea mosaic virus* (CPMV) moves whole virions through heavily modified plasmodesmata: their MP assembles into transport tubules, which penetrate through the plasmodesma (Van Lent *et al.*, 1990, 1991; Kasteel *et al.*, 1993; Wellink *et al.*, 1993; Kasteel *et al.*, 1997). Despite increasing knowledge about the functioning of the CPMV MP (Bertens *et al.*, 2000; Bertens *et al*., 2003; Carvalho *et al.*, 2003; Pouwels *et al.*, 2002) so far there is no information on host proteins which may specifically bind this protein during its targeting to the plasmodesma or during the subsequent tubule formation. However, it is anticipated that various host proteins will bind to CPMV MP, in analogy to observations with other plant viral MPs (Dorokhov *et al.*, 1999, Ham *et al.*, 1999, Chen *et al.*, 2000; Soellick *et al.*, 2000; Huang *et al.*, 2001; Lin & Heaton, 2001; Matsushita *et al.*, 2001). Firstly, it is tempting to assume that the assembly of MP copies to form the transport tubule requires host protein binding as these tubules are exclusively assembled at the plasma membrane and have an extracellular polarity (Chapter 2; Kasteel *et al.*, 1996; Pouwels *et al.*, 2002). Secondly, in Chapter 2 it has been shown that plasma membranes encaging the transport tubule assembled on infected cowpea protoplasts contain pectin methyl esterase (PME), an enzyme involved in cell wall dynamics and previously implicated in the TMV movement process (Dorokhov *et al.*, 1999; Chen *et al.*, 2000; Tzfira *et al.*, 2000). Furthermore, the CPMV MP has been demonstrated to bind GTP, possibly for energy supply during its functioning, but it does not have GTPase activity (Chapter 4). The MP may therefore require a host factor to achieve GTP hydrolysis.

The tubular structures consist of multimers of the MP that accumulate in punctate structures in the plasma membrane from where the tubules start to grow out (Pouwels *et al.*, 2002). The targeting of the MP to the peripheral punctate structures is not dependent on the cytoskeleton but also here involvement of host factors cannot be excluded.

Recently, for a number of plant viruses the interaction between their MP and host proteins have been studied to some extent, and these viruses include *Cucumber mosaic virus* (Ham *et al.*, 1999), *Tomato spotted wilt virus* (Soellick *et al.*, 2000), *Tobacco mosaic virus* (Dorokhov *et al.*, 1999; Chen *et al.*, 2000; Waigmann *et al.*, 2000, Kragler *et al.*, 2003; Lee *et al.*, 2003), *Cauliflower mosaic virus* (Huang *et al.*, 2001), *Turnip crinkle virus* (Lin & Heaton, 2001), *Tomato mosaic virus* (Matsushita *et al.*, 2001) and *Tomato bushy stunt virus* (Desvoyes *et al.*, 2002). In these investigations different techniques were applied including application of the yeast two-hybrid system and cDNA library screening.

In this study, an alternative strategy to identify host proteins that might interact with CPMV MP was employed. A plasma membrane fraction was isolated from host plant material and analysed for the presence of potentially MP-binding proteins using two independent protocols: blot-overlay assays and affinity chromatography. This approach led to the identification of two candidate host proteins involved in CPMV MP functioning.

MATERIAL AND METHODS

Plant culture, plasma membrane purification and protein solubilisation

Cowpea (*Vigna unguiculata* 'California Blackeye') and *Nicotiana benthamiana* plants were grown under controlled conditions (23 °C at night and 25 °C during the day, 16 h day, 50% relative humidity). Cowpea plants were collected 2 weeks and *N. benthamiana* plants 8 weeks after sowing.

A plasma membrane fraction was purified from both primary and secondary cowpea and *N. benthamiana* leaves by phase partitioning following the protocol described by Larsson *et al.*, (1994).

The PlusOne 2D CleanUp kit (Amersham) was used for selectively precipitating protein for 2D gel eletrophoretic analysis. The pellet was resuspended in IPG (Isoelectric Focusing System) buffer (8 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT). Protein amounts were estimated by the Bradford procedure (Bio-Rad) using BSA as a standard.

2D gel electrophoresis

Iso-electric focusing was performed on immobilized pH gradient (IPG) strips (7 cm non-linear pH 3-10 gradient, Amersham). The protein sample (90 μg) was loaded into the IPG strip by rehydration overnight. The following running conditions for

focussing were used: 100 V for 1h, 200 V for 1h, 500 V for 1h, 1000 V for 1h, 3,500 V for 2h45 min, 5,000 V for 2h, 8,000 V until 63,317 Vh was reached. The strip was subsequently equilibrated for 15 min in a solution containing 1% DTT, 50 mM Tris/HCl pH 8.8, 6 M urea, 30% glycerol and 2% SDS. A second equilibration step was performed for 15 min in the same solution, with 2.5% iodoacetamide instead of DTT. The second dimension was carried out on homogenous 12% SDS-PAGE gels. After electrophoresis at 20 mA for 1h, and then at 40 mA until the front reached the end of the gel, gels were silver stained or electroblotted on immobilon-P membranes. The overlay using purified CPMV-MP as probe was performed as described by Carvalho *et al.*, (2003).

Column purification

200 µg of CPMV MP was immobilized on 500 µl of Talon column, previously equilibrated with a 1:1 mix of TBS/ProFoundTM Lysis buffer (Pierce). Plasma membrane proteins (900 µg) were mixed with two vol. of TBS/ProFoundTM lysis buffer, and incubated for 2 h at 4 °C. Following incubation, the plasma membrane extract was loaded on the CPMV MP column and incubated for 2h at 4°C. The column was then washed with TBS/ProFoundTM Lysis buffer and eluted with 300 mM of imidazole in TBS/ProFoundTM Lysis buffer. The eluted fraction was loaded on 12% SDS-PAGE gel, stained with silver or coomassie blue or electro-blotted onto a PVDF Immobilon membrane. Controls consisted of plasma membrane extracts filtered in a similar protocol over a Talon column without the MP. A Far Western assay using purified MP as an overlay probe was done according to Carvalho *et al.*, (2003). The protein band that showed interaction with CPMV MP was cut out of the gel for sequencing using mass spectrometry.

Mass spectrometry

Protein bands from gels were excised, dried, and digested in gel with trypsin (Sequencing Grade Modified Trypsin, Promega), according to Shevchenko *et al.*, (1996). Tryptic peptides were extracted from the gel and loaded on to a capillary Atlantis C18 column (15 cm x 75 µm; Waters). Peptides were eluted using a 30 min gradient of 0.5% formic acid in water to 0.5% formic acid in 80 % acetonitril at a flow rate of 0.2 µl/min. The C18 column was connected to the electro-spray of a Q-Tof-2 mass spectrometer (Micromass) by a PicoTip (New Objective). The Q-Tof-2 mass spectrometer was programmed to determine the charge of the eluting peptides, and, if appropriate (i.e. 2+ or 3+), the Q-Tof-MS switched to the MSMS mode applying

collision-induced dissociation (CID). The resulting CID spectrum contained the sequence information for a single peptide.

The ProteinLynx Global Server (PLGS; MicroMass) module was used to interpret the MS/MS spectra and to generate amino acid sequences for the various tryptic peptides.

RESULTS

Detection of MP-binding proteins using a blot overlay assay

Two lines of evidence suggest that the CPMV MP may interact directly with plasma membrane proteins. Firstly, in infected tissue the MP is targeted to the periphery of the cell where it forms a plasmodesma-penetrating tubule at close proximity to the plasma membrane. Secondly, in infected cowpea protoplasts such tubules are also formed, extending from the cell surface, and remaining completely covered by the plasma membrane (see Chapter 2). Therefore, plasma membrane-enriched fractions were isolated from both CPMV-infected and uninfected cowpea, and from uninfected *N. benthamiana* (see Materials & Methods), and the proteins therein were separated by SDS/PAGE, blotted onto membrane and probed with CPMV MP. Fig. 1 shows that MP bound three proteins bands with a relative electrophoretic mobility corresponding to MWs of ~28 kDa, 30 kDa and 34 kDa in plasma membrane extracted from cowpea. Protein bands with similar MW were detected when plasma membrane fractions extracted from *N. benthamiana* leaves were analysed for their

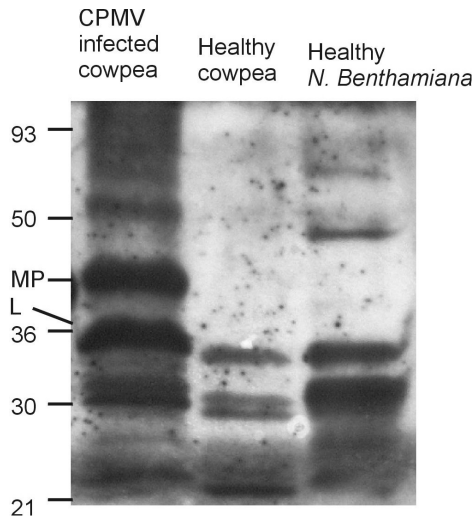

Figure 1 Identification of putative CPMV MP-binding host proteins using blot overlay assays. Blots with plasma membrane fractions isolated from CPMV-infected cowpea, uninfected cowpea and *Nicotiana benthamiana* were probed with purified MP. Positions of molecular mass markers, the MP and L protein are indicated on the left.

affinity to MP. In the plasma membrane extract from CPMV-infected cowpea, two extra bands were observed, with MW corresponding to the MP (48 kDa) and the large (L) coat protein of CPMV (37 kDa). To exclude possible cross reactivity of the CPMV MP antiserum with plasma membrane proteins, the same assay was performed without the MP in the overlay buffer. Apart from an expected MP-band in the infected cowpea lane, no binding of antibodies to other proteins was observed (data not shown).

Detection of MP-binding proteins using a blot overlay assay in combination with 2D gel electrophoresis

Two-D gel electrophoretic separation of the plasma membrane enriched protein fraction was performed to improve the subsequent detection of the MP-binding proteins using the overlay assay. Samples of cowpea plasma membrane fractions were separated in the first dimension using a non-linear pH gradient from 3 to 10. After running the second dimension (SDS-PAGE) the proteins were either stained with silver or blotted onto Immobilon P-membrane and probed with CPMV MP. In the silver-stained gel approximately 200 spots could be detected (Fig. 2A), of which some were very abundant. In the blot overlay assay of a parallel gel (Fig. 2B), only a limited number of spots showed affinity to MP. From a visual comparison of the silver stained protein pattern with the protein spots in the overlay gel, some spots in the silver stained gel could be identified as MP-binding proteins (indicated by arrows in Fig. 2A and B). The most prominent spot (encircled in Fig. 1A and B) was isolated from the silver

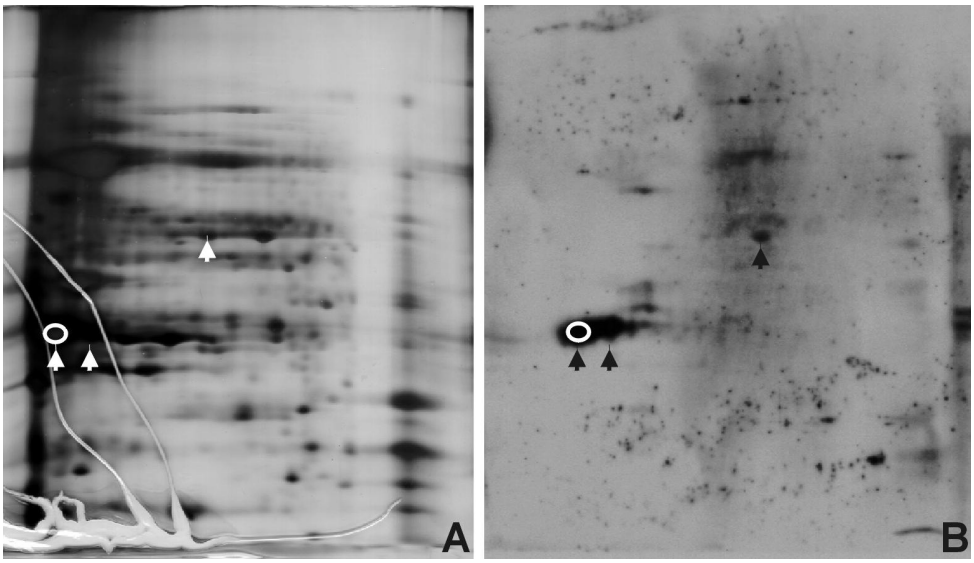

Figure 2 Isolation of a putative CPMV MP-binding host protein using 2D electrophoresis. (A) Silver-stained two-dimensional gel electrophoretic pattern of plasma membrane proteins extracted from cowpea leaves. (B) Overlay assay of a blot from a similar gel probed with purified MP. Proteins showing MP-binding and which could be traced in the silver-stained gel are indicated with an arrow. The encircled spot was isolated and sequenced.

stained gel and digested with trypsin for sequencing using mass spectrometry. From the MSMS data obtained 12 peptide sequences showed homology to the chlorophyll A-B binding protein from diverse plant species. The best match was found with the chlorophyll A-B binding protein from soybean (cowpea and soybean are closely related members of the family *Fabaceae*) (data not shown). The estimated MW of the spot in the 2-D gel (~25 kDa) is compatible with the size of chlorophyll A-B binding proteins. This observation indicated that the plasma membrane fraction used for the MP binding overlay assay was contaminated with chloroplast (thyllakoid) membranes. It is very unlikely that chlorophyll A-B binding protein is involved in the movement of CPMV. Therefore, it was concluded that the method described above, where by denatured plant proteins were tested for affinity to MP after immobilization onto a membrane is not specific enough for identifying the host protein(s) that *in planta* interact with MP.

Affinity chromatography of cowpea plasma membrane proteins

An alternative experimental approach was performed to investigate specific binding of cowpea plasma membrane proteins to MP using affinity chromatography with his-tagged MP immobilized to a Talon column. To this end, the cowpea plasma membrane protein fraction was loaded onto a MP column, and after washing, the bound proteins including the MP, were eluted with imidazole and further analysed. An

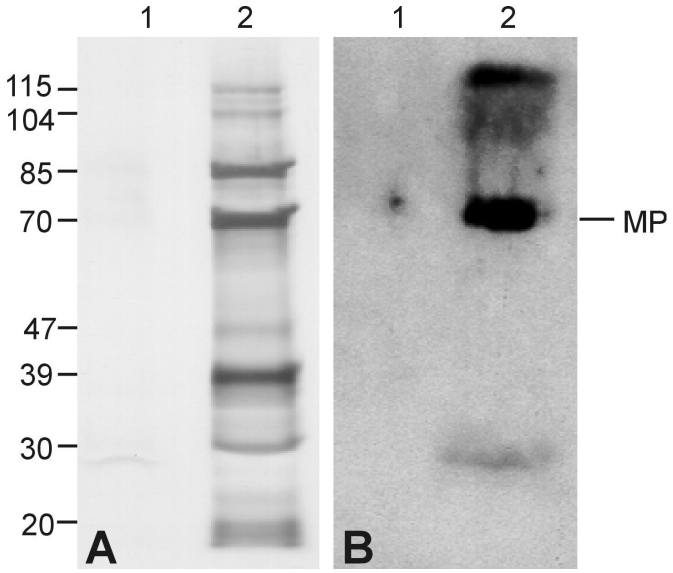

Figure 3 Identification of putative CPMV MP-binding host proteins using affinity chromatography. (A) Silver-stained gel showing proteins retained from a plasma membrane fraction by a Talon column with immobilized MP. (B) Blot from a similar gel probed in an overlay assay with purified MP. Molecular markes of the proteins are indicated on the left.

identical fraction was prepared from plasma membrane loaded onto a Talon column without the MP, to check if any of the proteins in the membrane fraction bound to the column material. SDS-PAGE followed by silver staining revealed that the MP-binding protein fraction contained eight major bands with apparent MW of 115, 104, 85, 70, 45, 39, 30 and 20kDa (Fig. 3A, lane 2). No proteins appeared to bind to the Talon column material (Fig. 3A, lane 1). The same fraction of affinity-bound proteins was also tested in the overlay assay to verify whether these proteins still showed affinity for MP. Two of the five major bands in the silver stained gel (30 and 70 kDa) and one minor band (125 kDa) were observed in the overlay blot (Fig. 3B). In a previous overlay experiment with unfractionated plasma membrane proteins (without affinity purification), two major bands with MW around 30 kDa showed affinity with MP (Fig. 1). As both the overlay assay on whole plasma membrane extracts and the affinity purification revealed this 30 kDa band, it was cut from an equivalent silver stained gel as depicted in Fig. 3A and used for sequence analysis.

Sequence analysis of the 30 kDa MP-binding plasma membrane protein

Using the two different experimental approaches a 30 kDa band showed consistent affinity for MP. Sequence analysis was performed on the 30 kDa protein band as obtained from the MP affinity column. The band was isolated from the gel, digested with trypsin, and the resulting peptides were analysed by MS/MS. Four peptide sequences deduced from the MSMS spectra (EQDVSLGANKFPER, DVSLGANKFPER, SLQANKFPER and FGDLSGGANFVA) showed identity with a 289 aa aquaporin protein of *Pisum sativum* (accession CAA38241.1), of which three sequences cover the same tryptic peptide (Fig. 4A), and also with plasma membrane residing aquaporin proteins of other legume plant species (*Medicago trunculata*, *Phaseolus vulgaris*). In the same digest, two peptides (RVNALENVVKPR and LENTISYIK) showed identity to vacuolar ATPase subunit D of *Arabidopsis thaliana* (Fig. 4B) (accession CAB46439.1), whereas two other peptides (IEYSMQLNASR and IVCENTLDAR) were identified with identity to vacuolar ATPase subunit E of *Oryza sativa* (Fig. 4C) (accession BAB85263.1). The proteins identified by MSMS analysis have molecular masses close to 30 kDa (aquaporin ~31 kDa; vacuolar ATPase subunits D and E ~27-29 kDa), indicating that the digested protein band represented a mixture of aquaporin and vacuolar ATPase subunits. From the MSMS data no conclusions can be drawn about the relative abundance of the identified proteins in the isolated bands.

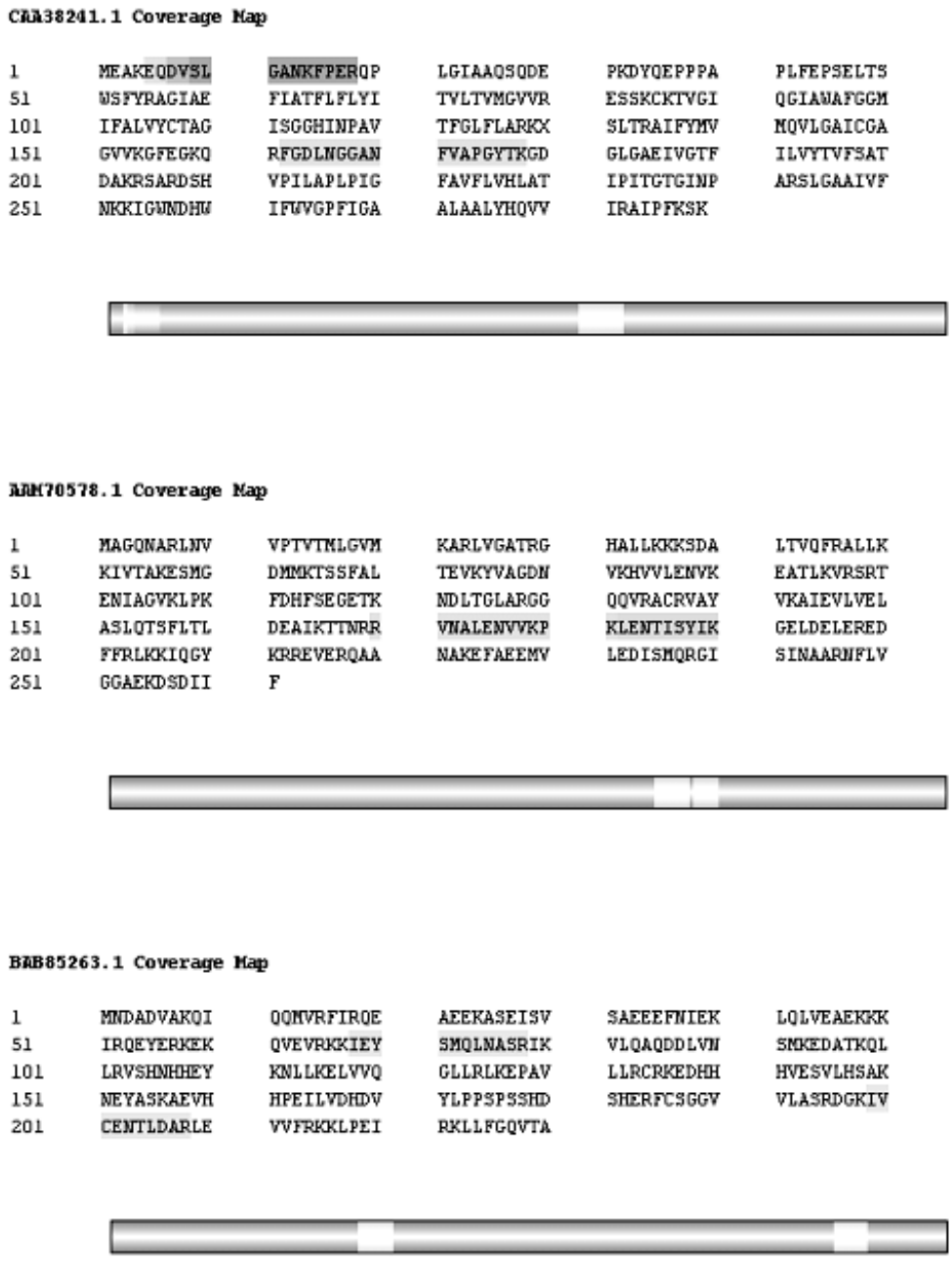

Figure 4 Amino acid sequence of eight tryptic peptides derived from the 30 kDa MP-interacting protein band separated after affinity chromatography and their alignment with (A) aquaporin protein from *Pisum sativum* (accession CAA38241.1), (B) vacuolar ATPase from *A. thaliana* (accession CAB46439.1), (C) vacuolar ATPase from *Oryza sativa* (accession BAB85263.1). The sequenced peptides are indicated in different colors.

DISCUSSION

Cell-to-cell spread of CPMV, as well as many other plant viruses, is mediated by virus-encoded MPs that are targeted to the plasma membrane where they assemble into transport tubules (Van Lent *et al.*, 1990, 1991; Kasteel *et al.*, 1993; Wellink *et al.*, 1993; Kasteel *et al.*, 1996). The molecular pathway by which CPMV MP promotes

cell-to-cell transport of virions is not fully understood, but the close association between MP and plasma membrane suggests an interaction of MP with plasma membrane proteins. Here we used both a blot-overlay assay and affinity column chromatography to identify proteins in the plasma membrane fraction of cowpea that were able to bind purified MP. Using either method a 30 kDa protein band was identified among the limited number of MP-binding host proteins. Both the membrane fraction of cowpea and *N. benthamiana* contained this band.

Subsequent sequence analysis revealed that the 30 kDa protein band as separated by SDS-PAGE actually consisted of a mixture of two proteins, an aquaporin-like protein and two subunits (D and E) of vacuolar ATPase. Protein database analysis revealed that these proteins have indeed sizes of approx. 30 kDa. The analysis did not reveal pectin methyl esterase (PME), an enzyme previously demonstrated (Chapter 2) to be present in plasma membrane-bound transport tubules produced on CPMV infected protoplasts. Apparently, this cell wall residing host enzyme with estimated MW of 37 kDa (Chen *et al.*, 2000) is not present in the plasma membrane fraction or does not physically interact with the MP.

Aquaporins are ubiquitous membrane channel proteins that facilitate and regulate the permeation of water across biological membranes (for recent reviews see Baiges *et al.*, 2002; Johansson *et al.*, 2000). Their physiological importance is revealed by their widespread occurrence in plants, animals, fungi and bacteria (Johanson *et al.*, 2001). Aquaporins are members of a family of membrane intrinsic proteins (MIP) and in the plant kingdom, a single plant expresses a large number of homologues, which can be subdivided in 4 groups, with highly conserved amino acids sequences and intron positions in each group. As four peptides analysed showed matches with plasma membrane intrinsic aquaporin proteins (PIP) only, one of the two proteins present in the 30 kDa protein band probably represents a cowpea homologue located in the plasma membrane. It is conceivable that *in planta* this aquaporin undergoes an interaction with the plasma membrane-targeted viral MP.

The same holds for vacuolar-type ATPases (V-ATPases), which are a universal class of proton pumps. Despite the name, V-ATPases operates not only at the tonoplast, i.e. the vacuole-cytoplasma interface. The V-ATPase complex is characterized by a membrane-bound segment (V_0) responsible for the translocation of protons from the cytoplasm into the vacuole, endoplasmic reticulum, Golgi and lysosomal vesicles, and a soluble portion (V_1) that supplies the energy for translocation by hydrolyzing ATP. Both V_0 and V_1 are made-up from a number of subunits (Grabe *et al.*, 2000; Maeshima, 2001). V-ATPases are involved in driving the active transport of ions and metabolites across membranes. In BHK-21 cells and MA104 cells respectively, the V-ATPase facilitates the *Semliki forest virus* and Rotavirus transport

by acidification of the endosomes and pumping protons in the Golgi apparatus (Palokangas *et al.*, 1994; Chemello *et al.,* 2002). As two peptides match V-ATPase subunit D and two match the subunit E, these protein species with comparable MWs apparently constitute two other proteins in the 30 kDa protein band. If MP immobilized on the Talon column binds V-ATPase through one of the subunits, it is conceivable that at least some of the other protein bands found in the eluted fraction (Fig. 3A) represent other subunits of the ATPase complex. Sequencing of the other protein bands would clarify this.

Given their intracellular localisation, both an aquaporin and a V-ATPase may be directly bound *in planta* by the viral MP. There are no reports describing a physical interaction between these two categories of host proteins (see above), hence they seem both to interact directly with the MP. Of course further studies should be done before a firm conclusion can be drawn that the interaction with aquaporin and/or V-ATPase is a functional one. For instance, (immuno)co-localisation studies *in planta* could be done. Another approach to verify the involvement of the two host proteins identified could be mutational analysis of the MP and demonstrating that loss of binding capacity to either of the two 30 kDa host proteins coincides with dysfunctionality of virus movement.

It may of course be criticised that the binding studies reported here are rather conventional, and that other reported approaches such as screening cDNA libraries using the yeast two-hybrid system should be performed. However, such approaches have frequently shown to end up with no results or false positive clones, while the host proteins identified so far using such methods often are questionable with respect to their potential function in virus movement. With CPMV MP as bait an extensive two-hybrid screening of a *N. benthamiana* cDNA library yielded no positive clones, while screening with *Alfalfa mosaic virus* MP yielded three candidate host proteins with unknown function (Zuidmeer, 2002). An Atp8 protein (Lin & Heaton, 2001) and MPI7 protein (Huang *et al.,* 2001) from *A. thaliana* were reported to interact with the MPs of *Turnip crinkle virus* and CaMV respectively. Two homologous NSm-binding proteins of the DnaJ family were identified in *N. tabacum* and *A. thaliana* (Soellick *et al.,* 2000). However, the functions of these movement protein interacting partners with respect to cell-to-cell movement have yet to be elucidated.

In our approach, two independent assays detected a 30 kDa protein band among a limited number of MP-binding cowpea proteins, and this band was also present in the MP-binding protein pattern of another host, *N. benthamiana*. It is also worthwhile mentioning here that both aquaporins and V-ATPases are well conserved among plant and animal species. The observation that the CPMV transport tubules are formed in protoplasts of non-host plants and even in insect cells (Kasteel *et al.,* 1996) fits the

idea that some host protein involved in MP targeting and in tubule formation should be strictly conserved.

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CHAPTER 6

General discussion

The aim of the research described in this thesis was to investigate the mechanism of cell-to-cell movement employed by *Cowpea mosaic virus* (CPMV), with special emphasis on the interactions between the virally encoded movement protein (MP), the virion proteins and host proteins.

In infected tissue, CPMV MP is targeted to the periphery of the cell where it forms a plasmodesma-penetrating tubule to facilitate the cell-to-cell transport of whole virions (Wellink & Van Kammen, 1989, Van Lent *et al*., 1990, 1991). In infected protoplasts these tubules are also formed at the cell surface and they are then completely covered by the plasma membrane (Van Lent *et al*., 1991, Kasteel *et al*., 1996). This phenomenon was exploited in Chapters 2 and 3 for initial studies on the interactions between MP, virus particle, and host proteins. An unexpected outcome of this analysis was that virions encaged by the movement tubule contain a hitherto unknown version of the small coat protein (S CP) with an electrophoretic mobility significantly slower than that of the S CP in virions isolated from the cytoplasm of infected cells. Two electrophoretic forms of the S CP are usually found in extracts from infected plants and in purified virus preparations, denoted S-s (low) and S-f (fast), the S-f being a proteolytically processed form of the unprocessed S-s (Taylor *et al*., 1999). The deviant mobility of the S CP could be the result of a post-translational modification of the protein such as phosphorylation and/or glycosylation. Recently, it has been demonstrated that neither CPMV CP is glycosylated (Altmann & Lomonossoff, 2000), despite an earlier publication claiming they were (Partridge *et al*., 1974). This points against glycosylation as reponsible modification, but it should be noted that in those studies total virion preparations were analysed, while the modification of S CP is only found in the (minority of) tubule-encaged particles. Alternatively, the S CP in these virions could be phosphorylated. This is particulary likely, since the MP is able to bind GTP (see Chapter 4) and this property could have a function in trapping the virion particles at the tubule assembly sites, as this process probably requires energy. It is conceivable that during the assembly of the transport tubule, MP-bound GTP is hydrolysed whereby the P is transferred to the S CP of the encaged virions, thus explaining its slower mobility in gels. With *Potato virus A* (PVA), a potyvirus, phosphorylation of the CP appears to be important for the formation and stability of viral ribonucleoprotein complexes and this has been suggested to function in the movement of PVA by controlling the stability of potyvirus movement intermediates (Ivanov *et al*., 2001, 2003). Irrespective of how CPMV S CP of tubule encaged virions have been modified, this distinction indicates that this

protein plays a role in the transport of the virions. However, *in vitro* blot overlay assays have demonstrated that this role is not MP-virion binding as it appears to be the L CP, which directly interacts with the MP (Chapter 3).

The ability to purify plasma membrane-bound tubules from infected protoplasts allowed immunological analysis of these complexes to identify plasma membrane proteins that may interact with the viral MP. Probing of these membrane-bound tubules with two different antibodies against pectin methylesterase (PME) indicated the presence of this enzyme. PME is known to be involved in cell wall turnover and its activity modulates pH and ion balance and affects cell wall rigidity (Micheli, 2001). Furthermore, the involvement of PME in movement of viruses is reinforced by the evidence that this enzyme interacts with the MPs of *Tobacco mosaic virus* (TMV), *Turnip vein clearing virus* (TVCV), and *Cauliflower mosaic virus* (CaMV) (a virus which also employs a tubule-guided movement mechanism) (Chen *et al*., 2000) and is involved in the exit of TMV particles from the vasculature (Chen & Citovsky, 2003). Cell wall modulation could well be a necessity for the modification of the cell wall around the plasmodesmal pore to enable the insertion of the viral transport tubule. Although for CPMV no noticeable dilation of the plasmodesmal pore has been reported, for *Alfalfa mosaic virus* (AMV) a nearly two-fold increase in diameter of plasmodesmata appears to be required for tubule-guided transport of the virions (Van der Wel *et al*., 1998).

Over the past few years, interactions between viral MPs and host proteins have been studied for several virus-host combinations and using a variety of techniques including the screening of host cDNA libraries by yeast two-hybrid analysis and blot overlay assays. Several host proteins potentially involved in virus cell-to-cell movement have been identified. Using the yeast two-hybrid system a tobacco protein (denoted 2bip) with so far unknown function was found to bind the MP of *Cucumber mosaic virus* (Ham *et al*., 1999), two homologous proteins of the DnaJ family from tobacco and *Arabidopsis* were found to bind the NSm protein of *Tomato spotted wilt virus* (Soellick *et al*., 2000), MP-binding *Arabidopsis* proteins with so far unknown functions were found to bind the MPs of CaMV (Huang *et al*., 2001) and *Turnip crinkle virus* (Lin & Heaton, 2001) and a homeodomain leucine-zipper protein was found to interact with MP of *Tomato bushy stunt virus* (Desvoyes *et al*., 2002). Far Western screening of a *Brassica campestris* cDNA library identified MIP102, a putative orthologue for a transcriptional coactivator KELP of *Arabidopsis thaliana*, to bind to the MP of *Tomato mosaic virus* (Matsushita *et al*., 2001), while PME was identified in overlay assays of cell wall extracts to bind the MP of TMV (Chen *et al*., 2000; Dorokhov *et al*., 1999). However, with the exception of PME, the function of all

these proteins in the host as well as their role in viral cell-to-cell movement has yet to be elucidated.

In view of these outcomes, in this thesis a different approach to identify MP-interacting host proteins was used. It was anticipated that the CPMV MP would interact with at least one or more plasma membrane-associated host proteins. Therefore, a plasma membrane-enriched fraction of cowpea leaves was prepared and next assessed for containing MP-binding proteins using two different methods: a blot overlay assay and affinity column chromatography.

In the overlay assays, a limited number of MP-binding proteins with sizes of 34, 30 and 28 kDa were detected, while the affinity chromatography approach revealed five MP-binding proteins including again a 30 kDa protein band. Sequence analyses indicated that this protein band contained two host proteins, an aquaporin and a vacuolar ATPase. The PME found in the tubule enriched-membrane (Chapter 2) was apparently not identified by either method, indicating that the PME is not present in the plasma membrane fraction or it does not physically interact with the MP.

Of course, the significance of both host proteins identified as being MP-binders in Chapter 5 needs to be further verified. However, the current knowledge of these classes of plant proteins does not contradict MP binding *in vivo* during the infection process. Aquaporins are membrane channel proteins that facilitate and regulate the permeation of water across biological membranes (Baiges *et al*., 2002). Additionally, vacuolar-type ATPases (V-ATPases) represent a universal class of proteins involved in driving the active transport of ions and metabolites across membranes (Maeshima, 2001). The intercellular localisation of aquaporin and V-ATPase implies a direct binding to the viral MP. It is also worthwhile mentioning here that both aquaporins and V-ATPase are well conserved among plant and animal species. The observation that the CPMV transport tubules are formed in protoplasts of non-host plants and even in insect cells (Kasteel *et al*., 1996) fits the idea that any host protein involved in MP targeting and in tubule formation should be conserved. However, further experiments should be performed to confirm the functional binding of aquaporin and V-ATP. For instance, (immuno-) co-localisation studies in infected plant tissues should be done as well as mutational analysis of MP to demonstrate that loss of binding with either protein correlates with a defect in viral cell-to-cell movement. Alternatively, transgenic plants with silenced aquaporin or V-ATPase genes could be used to verify involvement in CPMV movement. Using protoplasts rather than whole tissue in this approach, could circumvent a possible lethal effect on the host.

With respect to the questions of which domains in MP are involved in virion and host protein binding, the binding to the L CP has been further investigated (Chapter 3).

It was shown that a similar C-terminal deletion in the MP, which has previously been shown to prevent virion binding, also results in loss of L CP binding.

The study of functional domains of the CPMV MP was extended in Chapter 4. This chapter describes the ability of the viral MP to bind specifically to GTP, whereas a mutant (AM5) with a modified D-motif (amino acids V142 and D143 were substituted for alanines) did not, indicating that the D-motif is part of the rNTP-binding site, as suggested by Chen & Bruening (1992). As the D-motif is conserved throughout the "30K" MP superfamily (Melcher, 2000), it can be speculated that all these MPs possess a GTP-binding domain. Our further experiments indicate that the MP has no GTP-ase activity by itself, and hence may need a host factor to achieve this. Although the role of GTP binding in MP functioning is not yet understood, the cytoplasmic location of mutant AM5 MP in protoplasts implies a function in targeting MP to the plasma membrane or multimerization of MP. The latter would be an attractive possibility. As discussed above, the deviant (slow electrophoretic mobility) form of S CP found in virions that are encaged in the tubule may be explained by phosphorylation and if the GTP-binding activity of MP has something to do with this, one option could be that GTP hydrolysis by MP only occurs in the presence of virus particles (S CP acting as P acceptor), to regulate co-assembly of virions and MP tubule. Subsequently, in analogy with microtubules, GTP hydrolysis may destabilise the transport tubule enabling rapid breakdown in the next cell to release the virions for further infection. The requirement for breakdown of the tubules for successful spread of virus infection has recently been suggested (Pouwels *et al*., 2003). The CPMV MP not only has distinct functional domains involved in tubule formation (Bertens *et al*., 2000; Pouwels *et al*., 2003) and virion binding and host protein binding (this thesis). Chapter 4 indicates that this protein is also able to bind ssRNA and ssDNA, like *Broad bean wilt virus 2* (BBWV2) MP and several MPs of less related plant viruses (Citovsky *et al*., 1990, 1991; Osman *et al*., 1992; Schoumacher *et al*., 1992; Li & Palukaitis, 1996; Qi *et al*., 2002). The significance of this binding is not yet known, although it cannot be ruled out that long-distance (vascular) transport of CPMV occurs in the form of an MP-RNA complex. For *Cucumber mosaic virus* (CMV), another virus which requires the MP and the CP for the cell-to-cell movement (Canto *et al*., 1997), a mutant has been described which is unable to form tubules in protoplasts but which is still able to move systemically (Canto & Palukaitis, 1999). Unlike wildtype virus, this mutant, however, is unable to traffic through plasmodesmata interconnecting tobacco epidermal cells. These observations suggest that CMV may employ different, tissue-dependent mechanisms of intercellular movement and this may also be the case for CPMV.

In summary, the various observations described in this thesis may be incorporated into the following speculative model for the mechanism of cell-to-cell movement employed by CPMV. The MP, possibly in dimeric or multimeric form (Pouwels *et al*., 2003), binds with the L CP of a population of virions at the intracellular site of virus replication/assembly and the complex is targeted to the plasma membrane. How the MP is transported to the cell periphery is not yet understood, but actin filaments, microtubules and the endoplamic reticulum are not involved (Pouwels *et al*., 2002). The MP then anchors to plasma membrane residing protein complexes (aquaporin and/or ATPase), accumulates and subsequently polymerises at the plasmodesmal pore into a transport tubule, thereby encaging the virions. To ensure faithful filling of the tubule with virions GTP hydrolysis, required for energy delivery in this process, only takes place when virions (*in casu* S CP) are in close proximity. Hydrolysis of the MP-bound GTP at the same time destabilises the tubule structure, which in the neighbouring uninfected cell then disintegrates to release the virions for further infection of the cell.

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Summary

For systemic infection of a host plant, viruses multiply in the initially infected cell and spread to the neighbouring cells through plasmodesmata (cell-to-cell movement), to eventually reach the vascular system and use the phloem to spread to other plant parts (long-distance movement). To achieve cell-to-cell transport through plasmodesmata, these complex pores in the plant cell wall must be modulated to allow viral passage. Two major types of cell-to-cell transport have been described, movement of the viral genome in a non-encapsidated form, as exemplified by *Tobacco mosaic virus* (TMV), and "tubule-guided" movement of mature virus particles (virions), exemplified by *Cowpea mosaic virus* (CPMV). In both mechanisms one or more virally encoded movement proteins (MP) play an essential role in the targeting of infectious entities from the site of replication to the plasmodesmata, as well as in the subsequent modification of and transport through the modified pores. However, it is generally recognised that intercellular movement is a concerted effort of not only viral factors but also host factors, the knowledge of the latter being very scarce at the moment.

With CPMV, the MP polymerises within the plasmodesmal pore into a transport tubule, through which mature virions then are delivered into the neighbouring uninfected cell. Identical tubules are also formed in single plant protoplasts that are infected with CPMV or transfected with the MP gene alone, hence, in the absence of cell wall and plasmodesmata.

At the onset of the research presented in this thesis, no information about host proteins interacting with the CPMV MP was available. Such interactions were to be expected, for instance during the process of transport (targeting) of the MP from its site of synthesis to the periphery of the infected cell, the polymerisation process at the plasma membrane, and the structural modification of the plasmodesma. Thus, the research described in this thesis focused on the functioning of the CPMV MP with special emphasis on its interactions with virion proteins and host proteins.

For initial studies on these interactions the property of the CPMV MP to assemble into tubules on single cell protoplasts was exploited in Chapter 2. Thus it was shown that virus particles residing in the tubule contain a single deviant species of the small coat protein (S CP) that is larger than the two forms of S CP (S-s and S-f) which are consistently found in virus present in the cytoplasm of infected cells. The nature of the deviation is not known, but the exclusive presence of this deviant S CP in virions that are being transported suggests that the S CP is in some way involved in cell-to-cell movement.

Identification of host proteins in isolated tubule fractions by electrophoretic analysis was not successful, but a directed search for potential host proteins by Western blot analysis using specific antibodies indicated the presence of pectin methylesterase (PME) in the plasma membrane surrounding the tubule (Chapter 2). This protein has previously been implicated in cell-to-cell movement of other plant viruses, i.e. TMV, *Cauliflower mosaic virus* and *Turnip vein clearing virus*. The PME enzyme is involved in cell wall turnover and affects cell wall rigidity by modulating pH and ion balance. Such cell wall dynamics could be a necessity for the modification of the plasmodesmal pore to enable the insertion of a viral transport tubule.

The interaction between the MP and virion proteins was further investigated in Chapter 3. Protein overlay assays and ELISA showed that the MP binds only to its homologous virions and that it is the large (L) coat protein which is involved in this binding. Considering also the deviation found in the S CP of virions within the transport tubules, it is conceivable that both CPs play a crucial but different role in the cell-to-cell movement of CPMV. A C-terminal deletion in MP, which *in planta* results in a mutant virus defective in cell-to-cell movement and producing tubules devoid of particles, also resulted in the abolishment of L CP binding, thus validating the *in vitro* binding approaches.

The ability of the CPMV MP to bind nucleic acid and rNTP was analysed in Chapter 4. It is shown that MP binds rGTP but no other rNTPs, and by site-directed mutagenesis the GTP binding site was located within a sequence motif conserved among the MPs of tobamo- and comoviruses. The non-GTP-binding mutant MP exhibited disturbed intracellular targeting and tubule formation, suggesting that GTP binding may play a significant role in targeted transport and multimerization of the MP. It was also shown that the MP is capable of binding both ss-RNA and DNA, but not ds nucleic acids.

The studies on possible interactions between CPMV MP and host (plasma membrane) proteins were extended in Chapter 5. To identify potential MP-binding host proteins, purified MP was used as a probe in overlay assays and affinity column chromatography to assess plasma membrane proteins for their affinity to the MP. In the blot overlay assays, candidate MP-binding proteins with apparent sizes of 34, 30 and 28 kDa were detected. Further analysis of the cowpea plasma membrane fraction using affinity chromatography also revealed a limited number of eight MP-binding proteins including again a 30 kDa protein band. Sequencing of the 30 kDa protein band revealed that it actually represented a mixture of two protein species, i.e. an aquaporin and a vacuolar-type ATPase. A possible role of these host proteins in viral MP functioning is discussed in Chapter 5.

Finally, in the General Discussion (Chapter 6) the results obtained in this thesis research are discussed and integrated in a speculative model for the functioning of the CPMV MP, accommodating the different observed interactions with virion and host proteins.

Samenvatting

Teneinde een volledige infectie van een plant te bewerkstelligen, moeten plantenvirussen in staat zijn om zich vanuit een primair geïnfecteerde cel te verspreiden, allereerst naar de omliggende cellen (“cel-cel” transport) en vervolgens via het vaatweefsel naar andere delen van de plant (“lange-afstands” transport). Bij het transport van de geïnfecteerde cel naar de ongeïnfecteerde omliggende cellen, moet het virus de starre celwanden passeren en gebruikt daarbij de in deze wanden aanwezige plasmodesmata. Dit zijn complexe openingen in de celwand die zorgdragen voor cel-cel communicatie en transport van biomoleculen, maar die het transport van macromoleculen, zoals een viraal genoom of een compleet virusdeeltje, niet toelaten. Dit betekent dat virussen in staat moeten zijn om (de doorlaatbaarheid van) plasmodesmata te modificeren. Dit is een actief proces waarvoor virussen speciale eiwitten produceren, de “transporteiwitten”.

Er zijn in principe twee mechanismen van virustransport te onderscheiden. Bij een deel van de plantenvirussen, met het tabaksmozaïekvirus (TMV) als belangrijkste voorbeeld, vindt verspreiding plaats van het niet-ingepakte virale genoom door plasmodesmata waarvan de doorlaatbaarheid is verhoogd zonder dat er morfologisch zichtbare veranderingen in deze structuur optreden. Een ander deel van de plantenvirussen, met als meest bestudeerde voorbeeld het cowpeamozaïekvirus (CPMV), verspreidt zich in de vorm van complete virusdeeltjes, die via speciale transportbuizen door de plasmodesma gesluisd worden. In beide mechanismen spelen de speciale virale transporteiwitten een essentiële rol, zowel bij het transport van genoom (meestal RNA) of virus binnen de cel naar de plasmodesma, als bij de modificatie van de plasmodesma en het transport hierdoor naar de volgende cel. Ongetwijfeld zullen ook een aantal factoren van de plantencel bij deze mechanismen een cruciale rol spelen. Echter hierover is op dit moment zeer weinig bekend. Een van de doelstellingen van het hier beschreven onderzoek was dan ook om inzicht te krijgen in de interacties tussen het virale transporteiwit en factoren van de plant.

Tijdens het infectieproces van CPMV, het virus dat in dit onderzoek is gebruikt, polymeriseert het transporteiwit in de plasmodesma en vormt zo een transportbuis met daarin de virusdeeltjes die in de volgende cel worden vrijgelaten voor verdere infectie. In protoplasten (dit zijn geïsoleerde plantencellen zonder celwand en plasmodesmata), worden na infectie met CPMV identieke buizen gevormd, maar nu aan het oppervlak van de cel. Van deze laatste eigenschap is gebruikt gemaakt om de transportbuizen te isoleren en de samenstelling ervan vast te stellen. De resultaten van deze experimenten zijn beschreven in Hoofdstuk 2. Een opmerkelijke waarneming was dat de virusdeeltjes die in de transportbuizen zijn opgesloten, een bijzondere vorm van het

kleine manteleiwit bevatten die afweek van de twee tot dan toe beschreven vormen van dit manteleiwit zoals die voorkomen in virusdeeltjes die in het cytoplasma van de cel accumuleren. Het feit dat de afwijkende vorm van dit manteleiwit uitsluitend lijkt voor te komen in de voor transport bedoelde virusdeeltjes, suggereert dat dit kleine manteleiwit een belangrijke rol speelt bij het cel-cel transport van CPMV.

Verdere componentanalyse van de gezuiverde transportbuizen liet de aanwezigheid zien van een plantenenzym, het pectine methylesterase (PME), een eiwit dat al eerder was gevonden op basis van interacties met het transporteiwit van andere virussen (bv. naast TMV ook caulimo- en potyvirussen). Het enzym PME is in de plant betrokken bij regulering van o.a. de rigiditeit van de celwand en het is derhalve denkbaar dat verandering van de celwandstructuur rond plasmodesmata een vereiste is voor aanpassing (vergroting) van de opening van de plasmodesma om de virale transportbuis hierin te kunnen assembleren.

De in Hoofdstuk 2 verkregen aanwijzing over de mogelijke betrokkenheid van met name het kleine manteleiwit bij het transport van CPMV, was aanleiding om in Hoofdstuk 3 nader onderzoek te doen naar de interacties tussen het transporteiwit en de afzonderlijke manteleiwitten. D.m.v. *in vitro* eiwit bindingsproeven en immunologische analyses (ELISA) werd vastgesteld dat het transporteiwit uitsluitend bindt aan homologe virusdeeltjes en dat juist het grote manteleiwit en niet het kleine betrokken is bij deze binding. Blijkbaar speelt het kleine manteleiwit een andere rol in het transportproces dan binding met het transporteiwit. Dat de *in vitro* waargenomen binding tussen het transporteiwit en het grote manteleiwit ook *in vivo* plaatsvindt, werd bevestigd door te laten zien dat eenzelfde (C-terminale) deletie in het transporteiwit welke *in vivo* leidt tot een transportdeficiënt mutantvirus dat slechts lege buizen produceert, tevens de binding met dit manteleiwit teniet doet.

Naast binding aan het grote manteleiwit bleek het CPMV transporteiwit ook te binden aan rGTP en enkelstrengs vormen van RNA en DNA (Hoofdstuk 4). Door middel van mutagenese werd de GTP bindingsplaats binnen het transporteiwit gelokaliseerd in een specifieke aminozuurvolgorde (het zgn. D-motief) die geconserveerd is in de transporteiwitten van tobamo- en comovirussen. Een mutant die niet in staat was om GTP te binden bleek eveneens gestoord in intracellulair transport en in de vorming van transportbuizen. Dit doet vermoeden dat GTP-binding een belangrijke rol speelt bij de adressering en polymerisatie van het eiwit.

Tenslotte is in Hoofdstuk 5 een eerste aanzet beschreven tot identificatie van componenten in de plasmamembraan die een mogelijke interactie aangaan met het transporteiwit. Hierbij is gebruik gemaakt van gezuiverde plasmamembraanfracties en twee experimentele benaderingen: blot overlay assays en affiniteitchromatografie. In de blot overlay assays werden drie eiwitbanden, met moleculaire massa's van 34, 30 en

28 kDa, gevonden waaraan het transporteiwit kon binden, terwijl bij affiniteitchromatografie vijf eiwitbanden werden waargenomen, waaronder opnieuw een met grootte 30 kDa. De aminozuurvolgorden van tryptische peptiden van het 30 kDa eiwitmateriaal lieten zien dat deze twee eiwitten bevatte, een aquaporine en een V-ATPase. De mogelijke rol van deze eiwitten in het functioneren van het transporteiwit is in Hoofdstuk 5 verder uitgewerkt.

Tenslotte worden in Hoofdstuk 6 de experimentele resultaten van dit proefschrift in een bredere context geplaatst en uitgewerkt tot een speculatief model van het transportmechanisme van CPMV.

Resumo

Para infectar sistemicamente uma planta hospedeira, os vírus vegetais replicam seu genoma nas célula inicialmente infectada, são tranpostados para as células adjacentes através do plasmodesma (movimento célula-à-célula), para então atingir o sistema vascular da planta e, através do floema, serem transportados para outros orgãos vegetais (movimento à longa distância). Para que o transporte viral célula-à-célula ocorra, os plasmodesmas (poros complexos presentes na parede celular comunicando células adjacentes) devem ser modulados para permitir a passagem do vírus. Dois principais tipos de transporte intercelular existem: o movimento do genoma viral na forma não encapsidada, exemplificado por *Tobacco mosaic virus* (TMV), e o movimento de partículas virais (vírions) mediado por túbulos, exemplificado por *Cowpea mosaic virus* (CPMV). Nos dois mecanismos, a proteína de movimento (PM) codificada pelo genoma viral exerce um papel essencial na translocação das entidades infecciosas do sítio de replicação para o plasmodesma, bem como na modificação e transporte através desses poros. O movimento intercelular depende de fatores virais, aspecto largamente estudado para vários gêneros virais, bem como fatores do hospedeiro vegetal, sobre os quais informações científicas ainda são escassas.

No caso de CPMV, polímeros de PM formam um túbulo no plasmodesma, através do qual os vírions são transportados para a células adjacentes não infectadas. Túbulos idênticos são encontrados em protoplastos infectados com CPMV ou trasnfectados apenas com o gene que codifica a PM, na ausência da parede celular e plasmodesma.

No início das investigações apresentadas nesta tese, não havia nenhuma informação sobre proteínas do hospedeiro interagindo com a PM de CPMV disponível. Tais interações eram esperadas, por exemplo durante o processo de trasnporte da PM do seu sítio de síntese até a periferia da célula infectada, no processo de polimerizacão da PM, e na modificação estrutural do plasmodesma. Portanto, as investigações descrita nessa tese, concentraram-se no funcionamento da PM de CPMV com enfâse na sua interação com as proteínas virais e do hospedeiro.

A propriedade da PM do CPMV em formar túbulos em protoplastos foi explorada no Capítulo 2. Foi mostrado que as partículas residindo nos túbulos contêm uma forma anômala da capa protéica menor (S), sendo maior que as duas formas da capa protéica menor consistemente observadasem vírus presente no citoplasma de células infectadas. A natureza desses desvios/anomalia não e conhecida, mas a presença exclusiva da forma anômala da capa protéica menor nos vírus que são transportados pelos túbulos, sugeste que essa capa protéica está, de alguma forma, envolvida no movimento de célula-à-célula.

Não foi possível identificar proteínas do hospedeiro nas frações de tubúlos por análise eletroforética, mas a análise de Western blot, utilizando anticorpos específicos investigação de potenciais proteínas do hospedeiro presentes nos túbulos, indicaram a presença da pectin metlhylesterase (PME) na membrana plasmática envolvendo os túbulos (Capítulo 2). Essa proteína parece estar envolvida no movimento intercelular das viroses de plantas, por exemplo TMV, *Cawliflower mosaic virus* and *Turnip vein clearing virus*. A PME também está envolvida no transporte através da parede celular e afeta a ridez da parede celular através da modulação do pH e balaço dos íons. Tal dinâmica da parede celular poderia ser a necessidade para modificação do plasmodesma, premitindo a inserção do túbulo de transporte.

A interação entre a PM e as proteínas virais foi investigada no Capítulo 3. A interação entre a PM e apenas seu vírus homologo e sua capa protéica maior foi mostrada por Far Western e ELISA. Considerando também a anomalia encontrada na capa protéica menor dos vírus presentes nos túbulos, é provavel que as duas capas protéicas tem um rota crucial, mas diferente, no movimento intercelular do CPMV. Uma deleção do C-terminal da PM, na qual *in planta* resulta em um vírus mutante deficiente no movimento célula-à-célula e que produz túbulos sem partículas virais, também resultou na abolição da interação com a capa protéica maior, validando os experimentos *in vitro*.

A habilidade da PM do CPMV de interagir com ácidos nucléicos e rNTP foi analisada no Capítulo 4. Foi mostrado que a PM interage com rGTP e nenhum outro rNTPs, e por mutação de sítio dirigida, o sítio de interação do GTP foi localizado dentro da sequência conservada das PMs dos tobamo- e comovirus. A PM mutante que não interage com GTP apresentou uma distribuição e formação dos túbulos afetada, sugerindo que a interação com GTP exerce um papel importante no transporte e multimerização da PM. Também foi mostrado que a PM é capaz de interagir com RNA e DNA de fita simples, mas não com ácidos nucléicos de fita dupla.

Os estudos das possíveis interações entre a PM do CPMV e as proteínas do hospedeiro (membrana plasmática) foi estendido no Capítulo 5. Para identificar potenciais proteínas do hospedeiro que interagem com a PM , essa foi purificada e usada como sonda em Far Westerns e cromatografia de coluna para identificar as proteínas da membrana plasmática que tem afinidade pela PM. Proteínas candidatas a interagirem com a PM, com peso molecular de 34, 30 e 28 kDa foram identificadas no Far Western. Análises das proteínas da membrana plasmática do caupi, usando cromatografia de coluna, também revelou um limitado número de oito proteínas que interagem com a PM incluindo novamente a banda de 30 kDa. Sequenciamento dessa banda revelou que esta representa uma mistura de duas espécies de proteínas,

aquaporim e ATPase tipo-vacuolar. Uma possível rota para essas proteínas no funcionamento da PM do CPMV é discutido no Capítulo 5.

Finalmente, na Discussão Geral (Capítulo 6) são discutidos os resultdos obtidos nessa tese e um modelo especulativo do funcionamento da PM do CPMV incluindo as diferentes interações com as protéinas virais e do hospedeiro.

Curriculum Vitae

Claudine Márcia Carvalho was born on July 25th, 1973, in Lavras, MG, Brazil. After finishing high school she studied Agronomy at the Federal University of Lavras (UFLA) and graduated in 1997. During her stay at UFLA between 1992 and 1997, she was awarded scholarships to initiate her scientific career. Subsequently, she followed an MSc programme in the Science of Plant Pathology at the same university, obtaining her MSc degree in July 1999, under the supervision of Prof. Dr Antônia dos Reis Figueira. Her thesis dealt with biological, molecular and microscopic studies of *Coffee ringspot virus*. In October 1999 she was awarded a 4-year scholarship by CAPES (Brazil) to carry out her PhD at the Laboratory of Virology of Wageningen University, The Netherlands, under the supervision of Dr Ir Jan van Lent and Prof. Dr Rob Goldbach, within the research project that resulted in this thesis.

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