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Identification of distinct steps during tubule formation by the movement protein of *Cowpea mosaic virus*

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The movement protein (MP) of *Cowpea mosaic virus* (CPMV) forms tubules through plasmodesmata in infected plants thus enabling virus particles to move from cell to cell. Localization studies of mutant MPs fused to GFP in protoplasts and plants identified several functional domains within the MP that are involved in distinct steps during tubule formation. Coinoculation experiments and the observation that one of the C-terminal deletion mutants accumulated uniformly in the plasma membrane suggest that dimeric or multimeric MP is first targeted to the plasma membrane. At the plasma membrane the MP quickly accumulates in peripheral punctuate spots, from which tubule formation is initiated. One of the mutant MPs formed tubules containing virus particles on protoplasts, but could not support cell-to-cell movement in plants. The observations that this mutant MP accumulated to a higher level in the cell than wt MP and did not accumulate in the cell wall opposite infected cells suggest that breakdown or disassembly of tubules in neighbouring, uninfected cells is required for cell-to-cell movement.

INTRODUCTION

To move from cell to cell plant viruses cross the rigid cell wall through plasmodesmata. For this purpose most, if not all, plant viruses encode one or more movement proteins (MPs) that are involved in modification of the plasmodesma to enable transport of virus particles or viral RNA–protein complexes from the infected to the neighbouring, uninfected cell (reviewed by Lazarowitz & Beachy, 1999).

Cowpea mosaic virus (CPMV), the type member of the *Comoviridae*, moves from cell to cell in the form of virions through tubules assembled from the MP. It has a genome consisting of two positive-sense RNA molecules, called RNA1 and RNA2. Both RNAs are translated into polyproteins, which are processed by a viral protease. RNA1 encodes proteins involved in replication of viral RNA and RNA2 encodes the cofactor for RNA replication (CR), two coat proteins (CPs) and the MP (for review see Pouwels *et al.*, 2002a).

Immuno-electron microscopy studies showed that the CPMV MP is a major component of the tubular structures (van Lent *et al.*, 1990). Similar tubular structures are also formed on CPMV-infected protoplasts (van Lent *et al.*, 1991) and on protoplasts transiently expressing the MP (Wellink *et al.*, 1993a), showing that MP is the only viral protein required for tubule formation.

In protoplasts CPMV MP fused to the N terminus of the green fluorescent protein (MPfGFP) was shown to accumulate in peripheral punctate structures and in tubular structures extending from the surface of the protoplast (Pouwels *et al.*, 2002b). In epidermal plant cells MPfGFP was mainly detected in fluorescent spots in the cell wall, possibly representing short tubular structures in modified plasmodesmata, both in the infected cell and in the neighbouring uninfected cells, indicating that MP is able to transport itself from cell to cell (Gopinath *et al.*, 2003). MPfGFP, however, is not able to support cell-to-cell movement of CPMV, since tubules formed by MPfGFP are unable to incorporate virus particles (Gopinath *et al.*, 2003).

Previous studies have indicated the presence of several functional domains in the CPMV MP (Bertens *et al.*, 2000, 2003; Carvalho *et al.*, 2003; Lekkerkerker *et al.*, 1996). The C terminus of the MP was shown to be involved in the interaction with virus particles in the tubule and the remaining part of the protein was found to be required for tubule formation. Within this tubule-forming domain, several regions involved in targeting of MP to the cell periphery were identified (Bertens *et al.*, 2000). Furthermore, recent studies on targeting of MP indicated that the cytoskeleton and the secretory pathway are not involved in targeting MP to the cell periphery, but that the MP might reach the cell periphery through diffusion (Pouwels *et al.*, 2002b).

The aim of the present study was to further elucidate the different steps involved in tubule formation and the domains of the CPMV MP associated with these steps. Therefore we have introduced C- and N-terminal deletions as well as subtle point mutations in the MP. Fusions of the mutated MPs to GFP or the yellow fluorescent protein (YFP) were used to determine their subcellular localization both in protoplasts and in plant tissue. Furthermore, the ability of the mutated MPs to support CPMV cell-to-cell movement was addressed. The results allowed us to refine the existing model on subcellular targeting and tubule formation by CPMV MP (Bertens *et al.*, 2000). Furthermore, indications that breakdown or disassembly of tubules in neighbouring, uninfected cells is required for cell-to-cell movement were found.

METHODS

Construction of the mutant MPs. All mutations were introduced by PCR with specific oligonucleotides in one or more of the following vectors: pTMPfGFP (Gopinath *et al.*, 2003), pMON-MPfGFP (Pouwels *et al.*, 2002b), pMON-MPfYFP or pM19GFP7 (Gopinath *et al.*, 2000). None of the constructs used in this study contained any additional coding mutations as checked by sequencing.

Plasmid pTMPfGFP contains a full-length cDNA clone of CPMV RNA2, which, in addition to both coat proteins, encodes MP and the cofactor for replication (CR) fused to the N terminus of GFP (MPfGFP and CRfGFP) (Fig. 1A). pMON-MPfGFP and pMON-MPfYFP encode MP fused to the N terminus of GFP or YFP under the control of a double 35S promoter (Fig. 1B). pM19GFP7 contains a full-length cDNA clone of CPMV RNA2 in which the GFP gene is introduced flanked by two artificial cleavage sites (Fig. 1C).

For the construction of pMON-MPfYFP the MP gene was amplified from pMON-MP using specific primers, thereby introducing a *Bgl*II site and a *Nco*I site. This fragment was digested with *Bgl*II and *Nco*I and cloned into *Bgl*II/*Nco*I-digested pMON-YFP.

For introduction of the point mutations (Y42F, Y42D, S130A, S130D, S225A and S225D) the primer-extension overlap method was used (Ho *et al.*, 1989). A forward primer in the 35S promoter and a reverse primer in the nos terminator together with two partially complementary primers containing the desired mutation were used on template pMON-MP (pMM48 in Wellink *et al.*, 1993a). From the resulting PCR product the *Bgl*II–*Xho*I fragment was cloned into *Bgl*II/*Xho*I-digested pMON-MPfYFP or the *Bgl*II–*Bam*HI fragment was cloned into *Bgl*II/*Bam*HI-digested pTMPfGFP or pM19GFP7.

For the construction of the C-terminal deletion mutants ($MP^{1–227}$, $MP^{1–251}$, $MP^{1–276}$ and $MP^{1–295}$) specific reverse primers introducing a *Bam*HI site were used together with a forward primer in the 35S promoter on pMM48 as template. The resulting PCR products were digested with *Bgl*II and *Bam*HI and cloned into *Bgl*II/*Bam*HI-digested pTMPfGFP. The *Bam*HI site is located in the 3′-end of the MP coding region and therefore all MP C-terminal deletion mutants still contain the last 11 amino acids of the C terminus of MP, in contrast to the previously described C-terminal deletion mutants (Bertens *et al.*, 2003; Lekkerkerker *et al.*, 1996). The presence of these amino acids should not influence the localization of the mutants, since they are not required for targeting MP to the cell periphery or for tubule formation (Lekkerkerker *et al.*, 1996). Furthermore, these 11 amino acids are a convenient linker between GFP and the different MP deletion mutants.

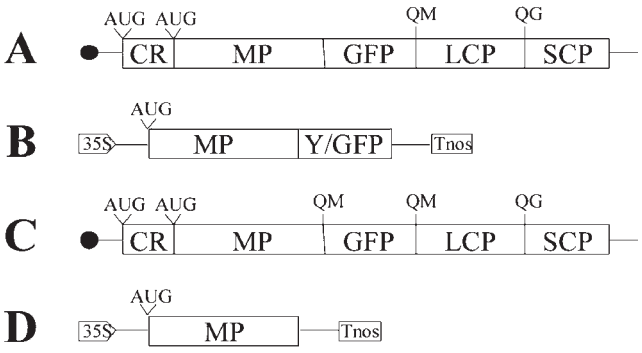

Fig. 1. Schematic representation of the RNA2 molecules and transient expression constructs used in this study. Bars indicate open reading frames, black circles represent VPg coupled to the N terminus of RNA, and the polyprotein cleavage sites are indicated with the recognition sites (QM and QG). (A) RNA2_MPfGFP is translated into two polyproteins due to the two in-frame start codons and therefore encodes MP and CR fused to the N terminus of GFP in addition to both coat proteins. (B) pMON-MPfYFP and pMON-MPfGFP are transient expression vectors encoding MPfYFP and MPfGFP respectively under the control of a double 35S promoter. (C) RNA2freeGFP encodes free GFP in addition to the other RNA2 proteins (CR, MP, LCP and SCP) due to the cleavage sites at both sites of GFP. (D) pMON-MP is a transient expression vector encoding MP under the control of a double 35S promoter. Abbreviations: CR, cofactor for RNA2 replication; MP, movement protein; LCP, large coat protein; SCP, small coat protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein.

For the construction of pMON-$MP^{\Delta3–12}$fGFP a reverse primer in the nos terminator was used with a specific primer on pMM48 as template, thereby introducing a *Bgl*II site and fusing the codons for amino acids 1 and 2 of the MP to the codon for amino acids 13. The resulting product was digested with *Bgl*II and *Xho*I and introduced in *Bgl*II/*Xho*I-digested pMON-MPfGFP.

For the construction of $pTMP^{\Delta1–4}$fGFP the 1·3 kb *Nar*I–*Bam*HI fragment of pTMΔAUG2 (Wellink *et al.*, 1993b) was cloned into *Nar*I/*Bam*HI-digested pTMPfGFP.

For construction of $pTMP^{AM2}$fGFP, $pTMP^{AM4}$fGFP and $pTMP^{AM6}$fGFP, the *Nar*I–*Bam*HI fragments from pTMAM2, pTMAM4 and pTMAM6 (all described by Bertens *et al.*, 2000) were cloned into *Nar*I/*Bam*HI-digested pTMPfGFP.

Inoculation of cowpea protoplasts and plants and their analyses. Protoplasts were isolated from cowpea (*Vigna unguiculata* L. 'California Blackeye') leaves and transfected by polyethylene glycol-mediated transformation as described previously (van Bokhoven *et al.*, 1993). When indicated, cerulenin was added to a final concentration of 50 μM immediately after addition of protoplast medium. Protoplasts were then incubated at 25 °C under continuous illumination. For inoculation of leaves, protoplast extracts were applied manually onto carborundum-dusted upper surfaces of primary leaves of 12-day-old cowpea plants. The protoplast extracts were made by resuspension of the protoplasts in 100 μl PBS and straining through a 0·45 mm syringe 10–15 times. For the plasmolysis experiments leaf segments of infected cowpea (approximately 1 × 2 cm) were submerged in a 1 M mannitol solution under vacuum for 5 min. For

imaging inoculated protoplasts or leaves a Zeiss LSM 510 confocal microscope was used with standard filters to visualize GFP and YFP. Western blot analysis and electron microscopy were performed as described previously (Bertens *et al.*, 2000; van Lent *et al.*, 1991).

RESULTS

Deletion mutants reveal distinct steps in the formation of tubular structures by CPMV MP

To determine whether distinct regions in the N or C terminus of CPMV MP are involved in targeting to peripheral punctate structures and tubule formation, several new N-terminal and C-terminal deletion mutants were constructed (Table 1). To visualize the mutant MPs, the mutations were introduced into a CPMV RNA2 variant expressing MP fused to the N terminus of GFP (RNA2_MPfGFP, Fig. 1; Gopinath *et al.*, 2003). Apart from MPfGFP, RNA2_MPfGFP also expresses CRfGFP, which is exclusively visible in the nucleus (Kasteel *et al.*, 1993), and both coat proteins (Fig. 1). Cowpea protoplasts or cowpea epidermal leaf cells were infected with RNA1 and wt or mutant RNA2_MPfGFP (we will only mention the name of the RNA2) and the location of MPfGFP was determined by confocal laser scanning microscopy (CLSM).

At 24 and 48 h post-inoculation (p.i.), protoplasts expressing MP^{WT}fGFP showed fluorescence in the nucleus (due to CRfGFP), in peripheral punctate structures and in tubules, extending from the plasma membrane into the culture medium (Fig. 2A; Gopinath *et al.*, 2003). All observations of protoplasts infected with CPMV, encoding wt or mutant MPfGFP, were done at 48 h p.i. unless stated otherwise. Fluorescence in protoplasts expressing $MP^{1–295}$fGFP (deletion of amino acids 296–331) accumulated in the nucleus, peripheral punctate structures (Fig. 2B, Table 1) and occasionally in short tubular structures (data not shown), indicating that deletion of amino acids 296–331 inhibited, but did not completely block, tubule formation. In the majority of the protoplasts expressing $MP^{1–276}$fGFP (deletion of amino acids 277–331), fluorescence was detected in peripheral punctate structures (Fig. 2C) and in some protoplasts fluorescence accumulated uniformly in the plasma membrane (data not shown). These results suggest that amino acids between position 277 and 295 are required for tubule formation but have only a minor function in accumulation in peripheral punctate structures and are not required for targeting to the plasma membrane. Results obtained with $MP^{1–251}$fGFP (deletion of amino acids 252–331) suggested that MP is targeted to the plasma membrane before it accumulates in peripheral punctate structures since in most infected protoplasts fluorescence

Table 1. Functional analysis of mutant CPMV movement proteins

MP mutant	Plasma membrane targeting*	Punctate structure formation*	Tubule formation†	Complementation by MP^{WT}†	Cell-to-cell movement‡
Wild-type	+	+	+	ND	+
1–295	+	+	±§	+	ND
1–276	+	±§	−	+	ND
1–251	+	−	−	−	ND
1–227	−	−	−	−	ND
Δ1–4	−	−	−	−	−‖
Δ3–12	−	−	−	+	ND
AM2	−	−	−	±¶	−
AM4	−	−	−	−	−
AM6	−	−	−	−	−
Y42F	−	−	−	+	−
Y42D	−	−	−	+	−
S130A	+	+	+	ND	−
S130D	−	−	−	+	−
S225A	+#	+#	+#	ND	+#
S225D	−	−	−	+	−

*Data obtained with protoplasts and epidermal leaf cells.

†Data obtained with protoplasts.

‡Data obtained with epidermal leaf cells using CPMVfreeGFP.

§Less efficient than MP^{WT}.

‖As determined by Wellink *et al.* (1993b).

¶Only very rarely tubules were observed.

#Delayed compared to WT.

ND, Not determined.

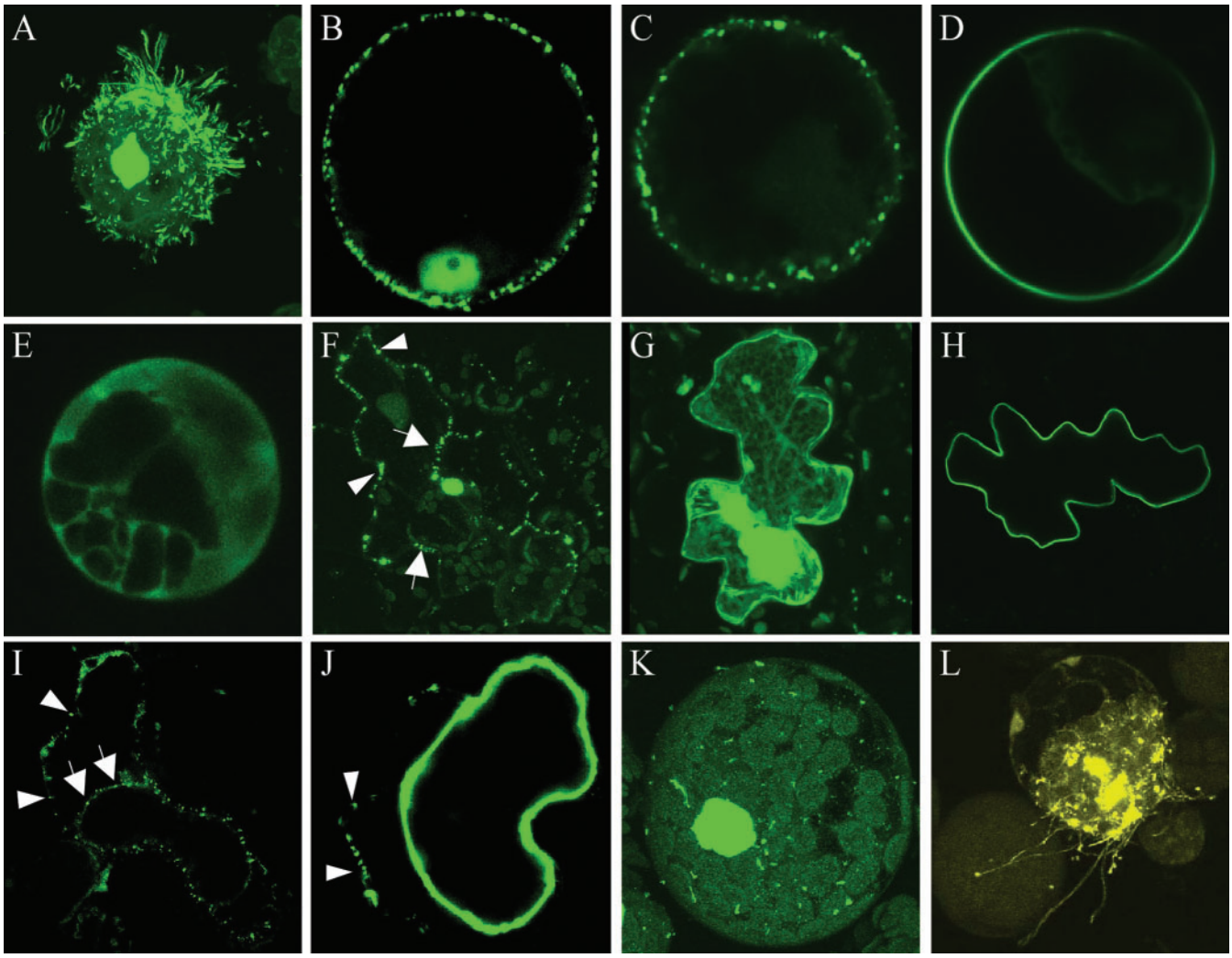

Fig. 2. Localization of wt and mutant CPMV MP in cowpea protoplasts (A–E, K, L) and cowpea epidermal leaf cells (F–J). Images shown are confocal fluorescence micrographs of a projection of serial optical sections (A, F, G, K, L) or a single optical section (B–E, H–J). (A–E) Protoplasts infected with RNA1 and RNA2_MP^{WT}fGFP (A), RNA2_MP$^{1-295}$fGFP (B), RNA2_MP$^{1-276}$fGFP (C), RNA2_MP$^{1-251}$fGFP (D) or RNA2_MP$^{1-227}$fGFP (E). (F–H) Epidermis cells infected with RNA1 and RNA2_MP^{WT}fGFP (F), RNA2_MP$^{1-227}$fGFP (G) or RNA2_MP$^{1-276}$fGFP (H). (I, J) Plasmolysed epidermis cells infected with RNA1 and RNA2_MP^{WT}fGFP (I) or RNA2_MP$^{1-276}$fGFP (J). (K) Protoplast infected with RNA1 and RNA2_MP^{S225A}fGFP. (L) Protoplast transfected with pMON-MP^{S225D}fYFP and pMON-MP^{WT}.

accumulated uniformly in the plasma membrane (Fig. 2D). Only in about 10 % of the transfected protoplasts was fluorescence observed in peripheral punctate structures in addition to the plasma membrane, indicating that amino acids between position 252 and 276 play a role in the formation of peripheral punctate structures, although other regions of MP are also important for this step. In protoplasts expressing MP$^{1-227}$fGFP, MP$^{\Delta 1-4}$fGFP and MP$^{\Delta 3-12}$fGFP (deletion of amino acids 228–331, amino acids 1–4 and amino acids 3–12 respectively), fluorescence accumulated in the cytoplasm (Table 1, Fig. 2E shows only MP$^{1-227}$fGFP), indicating that amino acids between position 228 and 250, position 1 and 4 and position 3 and 12 are essential for targeting MP to the cell periphery.

To determine whether the localization of the mutant MP–GFP fusions in epidermal cells was similar to that in protoplasts, cowpea leaves where inoculated with RNA1 and wt or mutant RNA2_MPfGFP. As shown previously (Gopinath *et al.*, 2003), MP^{WT}fGFP accumulated in spots in the cell wall of both infected (Fig. 2F, arrows) and neighbouring uninfected (Fig. 2F, arrowheads) cells, which probably represent short tubular structures in modified plasmodesmata and might correspond to the peripheral punctate structures and tubules formed in protoplasts (Fig. 2A). Except for MP$^{1-276}$fGFP the location of the N- and C-terminal deletion mutants in epidermis cells was similar to that in protoplasts (Fig. 2G shows only MP$^{1-227}$fGFP). MP$^{1-276}$fGFP accumulated uniformly in

the plasma membrane and not in peripheral punctate spots (Fig. 2H), like in the majority of the protoplasts (Fig. 2C). The observation that $MP^{1–276}$fGFP does not accumulate in spots in the cell wall of neighbouring cells opposite the infected cell (Fig. 2H) could indicate that $MP^{1–276}$fGFP accumulates to a higher level in the infected cell, causing accumulation of $MP^{1–276}$fGFP in the plasma membrane, thereby masking the spots. To determine whether $MP^{1–276}$fGFP accumulated in spots associated with plasmodesmata in the cell wall, plasmolysis experiments were performed on epidermis cells infected with RNA2_MP^{WT}fGFP (Fig. 2I) and RNA2_$MP^{1–276}$fGFP (Fig. 2J). As expected, in plasmolysed epidermis cells MP^{WT}fGFP accumulated in spots in both the plasma membrane (Fig. 2I, arrows) and cell wall (Fig. 2I, arrowheads). The plasmolysed epidermis cells infected with RNA2_$MP^{1–276}$fGFP showed similar fluorescent spots in the cell wall (Fig. 2J, arrowheads) in addition to strong labelling of the plasma membrane, indicating that $MP^{1–276}$fGFP indeed accumulates in spots in the cell wall.

The results described above suggest distinct steps during tubule formation and indicate that specific regions of MP are involved in these different steps.

Identification of functional domains using MP point mutations

Previous work on the localization of MP mutants relied on immuno-localization and fractionation experiments (Bertens *et al.*, 2000; Lekkerkerker *et al.*, 1996). However, the experiments with the GFP-fused deletion mutants clearly show that differences in the localization of mutant MPs can be very subtle and difficult to determine with immuno-localization and fractionation. Therefore, several new and previously described (Bertens *et al.*, 2000) mutations in amino acids, conserved between the MPs of comoviruses, were introduced in RNA2_MPfGFP (Fig. 1A) to determine the role of these amino acids in subcellular targeting of MP in both protoplasts and epidermal leaf cells (Table 1). To determine the effect of these mutations on cell-to-cell movement, the mutations were introduced in CPMVfreeGFP, expressing free GFP in addition to the other viral proteins (Fig. 1C), since tubules formed by MPfGFP are unable to incorporate virus particles and therefore do not support cell-to-cell movement (Gopinath *et al.*, 2003).

Most of the mutants were not targeted to the plasma membrane and accumulated in the cytoplasm. However, some interesting patterns were observed (Table 1). Mutating the conserved serine at position 225 into an alanine inhibited, but did not abolish completely, the formation of peripheral punctate structures and tubules, since protoplasts expressing MP^{S225A}fGFP showed a reduced number of peripheral punctate structures and fewer and shorter tubules compared to protoplasts expressing MP^{WT}fGFP (Fig. 2K). At 24 h p.i., when MP^{WT}fGFP had to some extent accumulated in peripheral punctate structures and short tubules, MP^{S225A}fGFP was not visible yet at the plasma membrane (data not shown), indicating a role for S225 in targeting MP to the plasma membrane. In infected epidermal leaf cells, like MP^{WT}fGFP (Fig. 2F), MP^{S225A}fGFP accumulated in spots in the cell wall of both the infected cell and the neighbouring uninfected cells, although the spreading seemed less extensive than for MP^{WT}fGFP (data not shown). In accordance with the localization pattern, CPMV expressing free GFP and containing MP^{S225A} ($CPMV^{S225A}$freeGFP) was able to move from cell to cell considerably slower than $CPMV^{WT}$freeGFP; using a hand-held UV-lamp green fluorescent spots were first observed on infected leaves 6–7 days p.i. for $CPMV^{S225A}$freeGFP and 3 days p.i. for $CPMV^{WT}$freeGFP. MP^{S225D}fGFP, in which S225 was replaced with the big, negatively charged aspartic acid (MP^{S225D}) instead of the small neutral alanine, was unable to target to the plasma membrane and accumulated in the cytoplasm both in protoplasts and epidermal cells (data not shown), confirming a role for S225 in targeting MP to the plasma membrane. As expected, $CPMV^{S225D}$freeGFP was confined to single infected cells.

To determine whether MP–MP interaction is involved in targeting MP to the cell membrane and formation of peripheral punctate structures, coinoculation experiments of cytoplasmic mutant MPs and wt MP were performed. Since the percentage of fluorescent protoplasts after infection with RNA2_MPfGFP is rather low (1–5 %), the mutations were introduced in pMON-MPfYFP, which expresses the MP fused to the N terminus of YFP under the control of a double 35S promoter (Fig. 1B). The transfection percentage with these constructs is much higher (10–70 %), which increases the chance of coinoculation, and the location of transiently expressed mutant MPs was similar to the virally expressed mutant MPs (data not shown). In all transient expression experiments, protoplasts were incubated in the presence of 50 μM cerulenin, an inhibitor of *de novo* lipid synthesis and protein acylation (Packter & Stumpf, 1975; Schneider *et al.*, 1993), which increased the number of tubules and their length as well as the total amount of MP^{WT}fYFP that accumulated in the protoplasts, as judged by fluorescence intensity and Western blotting, without having any other effect on the localization (data not shown).

For the coinoculation experiments protoplasts were transfected with mutant pMON-MPfYFP and pMON-MP^{WT}, encoding MP^{WT} under the control of a double 35S promoter (Fig. 1D, pMM48 in Wellink *et al.*, 1993a). Several of the cytoplasmic mutant MPs (Δ3–12, AM2, Y42D, Y42F, S130D and S225D) were complemented in both targeting to peripheral punctate structures and tubule formation (Table 1, Fig. 2L shows only S225DfYFP), indicating that MP–MP interaction is involved in targeting to the plasma membrane. Other cytoplasmic mutants were not complemented indicating that these mutant MPs have lost their ability to interact with other MP molecules or are not folded correctly (Table 1).

Degradation of MP might play a role in cell-to-cell movement

When protoplasts were infected with RNA2_MP^{S130A}fGFP fluorescence was observed in peripheral punctate structures and tubules (Fig. 3A), similar to MP^{WT}fGFP (Fig. 2A). However, unlike MP^{WT}fGFP, MP^{S130A}fGFP also accumulated uniformly in the plasma membrane (Fig. 3B) and the total amount of fluorescence observed in MP^{S130A}fGFP-expressing protoplasts seemed to be higher than in MP^{WT}fGFP-expressing protoplasts (data not shown). To confirm that the enhanced fluorescence intensity indeed corresponded to higher protein levels, equal amounts of protoplasts were transfected with equal amounts of pMON-MP^{WT}fYFP or pMON-MP^{S130A}fYFP, incubated for 24 h in the absence of cerulenin and analysed on a Western blot. The experiment was performed three times with different batches of protoplasts and a representative blot is shown in Fig. 4. A control staining with Ponceau showed that comparable amounts of protein were loaded (data not shown). The data indicated that the S130A mutation indeed increased the stability of the MP since MP^{S130A}fYFP (lanes 2 and 5) accumulated to a higher level in protoplasts than MP^{WT}fYFP (lanes 1 and 4) or other mutant MPs fused to YFP (data not shown). Higher amounts of MP^{S130A}fYFP were present both in the membrane fraction (P30, compare lanes 1 and 2) and the cytoplasmic fraction (S30, compare lanes 4 and 5). Also, the intensity of the additional bands, probably breakdown products of MPfYFP (Pouwels *et al.*, 2002b), was higher for MP^{S130A}fYFP compared to MP^{WT}fYFP. These additional bands were mostly present in the cytoplasmic fraction. Previously it has been shown that brefeldin A (BFA), which inhibits transport of vesicles from the endoplasmic reticulum to the Golgi, increases MP^{WT} protein levels in protoplasts, suggesting that CPMV MP is degraded in a pathway linked to the secretory pathway

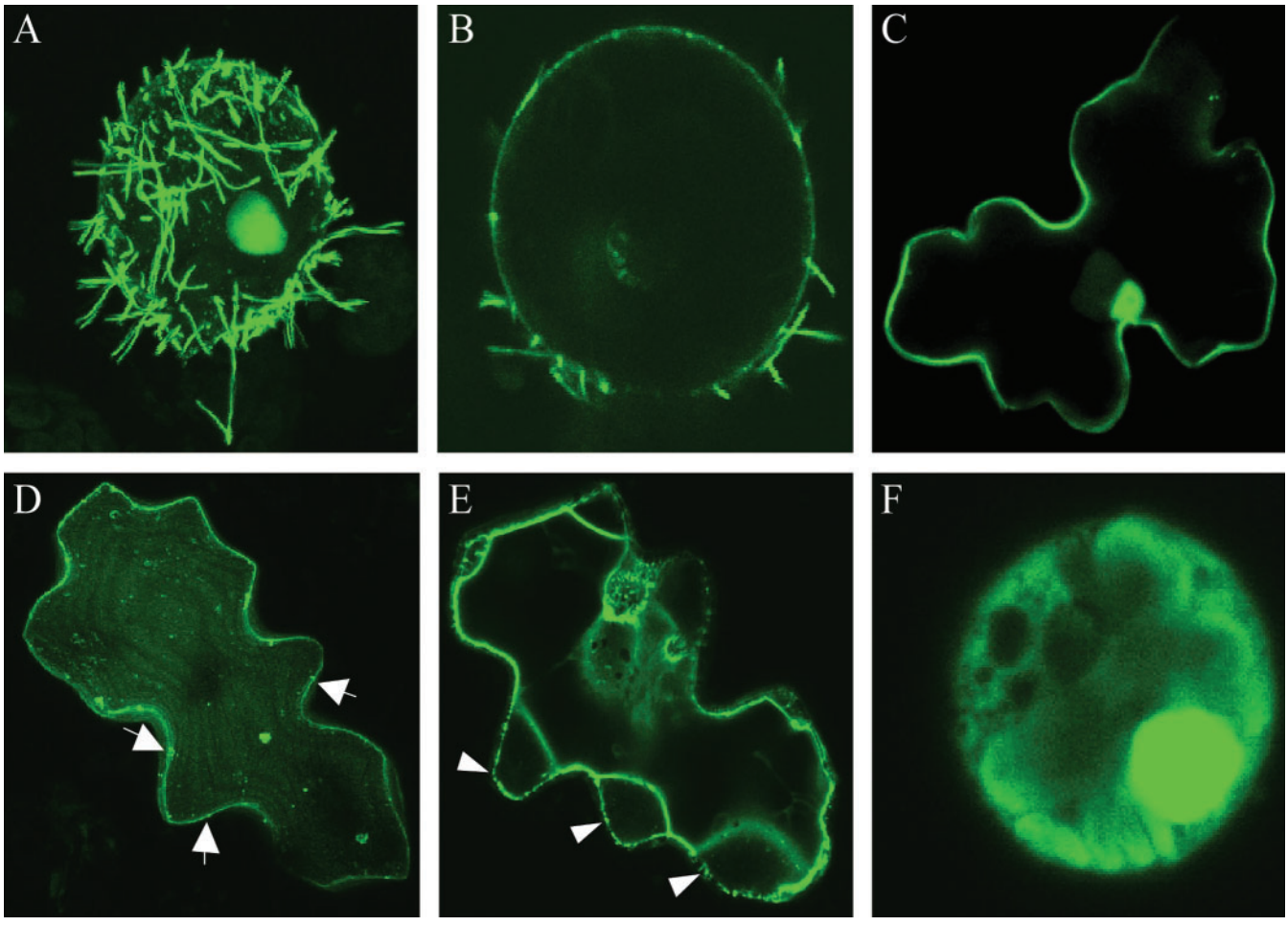

Fig. 3. Localization of MP mutated at position S130 in protoplasts and cowpea epidermal leaf cells. Images shown are confocal fluorescence micrographs of a projection of serial optical sections (A, D) or a single optical section (B, C, E, F). Protoplasts (A, B) and epidermis cells (C, D) infected with RNA1 and RNA2_MP^{S130A}fGFP. (E) Plasmolysed epidermis cell infected with RNA1 and RNA2_MP^{S130A}fGFP. (F) Protoplast infected with RNA1 and RNA2_MP^{S130D}fGFP.

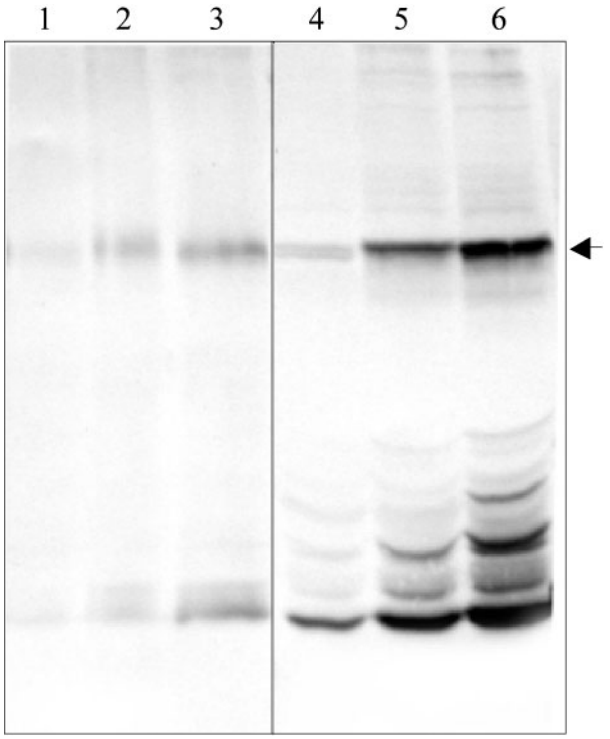

Fig. 4. Western blot analysis to compare the accumulation levels of MP^{S130A}fYFP, MP^{S130D}fYFP and MP^{WT}fYFP in protoplasts. Protoplasts were transfected with pMON-MP^{WT}fYFP, pMON-MP^{S130A}fYFP or pMON-MP^{S130D}fYFP and 24 h p.i. protoplast extracts were centrifuged at 30 000 ***g*** to obtain the membrane (P30) and cytoplasmic (S30) fraction. The samples were separated on a 10 % SDS-polyacrylamide gel and blotted onto nitrocellulose, which was then treated with an antiserum against MP. In lanes 1–3 25 % of the P30 fractions of protoplasts transfected with pMON-MP^{WT}fYFP (1), pMON-MP^{S130A}fYFP (2) or pMON-MP^{S130D}fYFP (3) were loaded compared to 100 % of the corresponding S30 fractions in lane 4–6. Arrow indicates MPfYFP.

(Pouwels *et al.*, 2002b). Incubation of MP^{S130A}fYFP expressing protoplasts with BFA, however, did not cause an increase in MP^{S130A}fYFP expression levels, as judged by fluorescence intensity (data not shown).

Unexpectedly, MP^{S130A} was not able to support cell-to-cell movement, since $CPMV^{S130A}$freeGFP was always confined to single infected epidermis cells. Analysis of the tubules formed on protoplasts infected with $CPMV^{S130A}$freeGFP by electron microscopy (Fig. 5B), revealed that these tubules were indistinguishable from tubules formed by MP^{WT} (Fig. 5A), both with respect to structure and content (virus particles). To determine whether the inability of MP^{S130A} to support cell-to-cell movement is due to mistargeting in epidermis cells, cowpea leaves were infected with RNA2_MP^{S130A}fGFP. In contrast to epidermis cells expressing MP^{WT}fGFP, which showed fluorescent spots in the cell wall of both the infected and neighbouring, uninfected cells

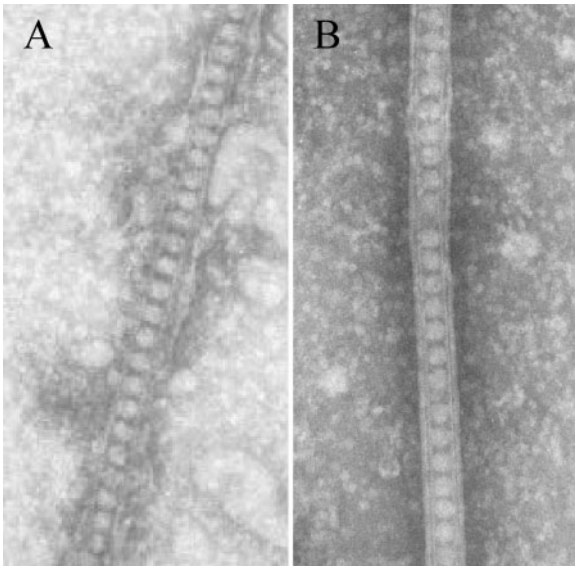

Fig. 5. Electron microscope images of tubules formed by MP^{WT} (A) and MP^{S130A} (B) on protoplasts.

(Fig. 2F), most epidermis cells expressing MP^{S130A}fGFP showed only a bright, uniformly labelled, fluorescent plasma membrane and no distinct spots (Fig. 3C). However, in some epidermis cells a fluorescent plasma membrane and fluorescent spots in the cell wall were observed (Fig. 3D, arrows). These differences might be due to variation in expression levels of MP^{S130A}fGFP as in cells with a high expression level of MP^{S130A}fGFP the plasma membrane might become saturated with MP^{S130A}fGFP, thereby obscuring the fluorescent spots. To determine whether MP^{S130A}fGFP accumulated in spots in the cell wall, plasmolysis experiments were performed on epidermis cells infected with RNA2_MP^{S130A}fGFP (Fig. 3E). In plasmolysed epidermis cells MP^{WT}fGFP accumulated in spots in both the plasma membrane and cell wall (Fig. 2I). The plasmolysed epidermis cells infected with RNA2_ MP^{S130A}fGFP showed similar fluorescent spots in the cell wall (Fig. 3E, arrowheads) and strong labelling of the plasma membrane, indicating that mutating S130 into alanine did not disrupt the ability of MP to target to spots (probably plasmodesmata) in the cell wall. However, unlike MP^{WT}fGFP (Fig. 2F), MP^{S130A}fGFP did not accumulate in the plasma membrane and/or spots in the cell wall of neighbouring cells (Fig. 3C, D), indicating that MP^{S130A}fGFP was not able to transport itself from cell to cell.

The experiments with MP^{S130A} indicate that S130 plays a role in cell-to-cell movement but is not involved in targeting the MP to the plasma membrane, accumulation in peripheral punctate structures and in tubule formation. One possible explanation for these observations is that the tubules, made by MP^{S130A}, cannot be disassembled in the neighbouring, uninfected cells, thereby blocking release of virions and consequently cell-to-cell movement. The

observations that MP^{S130A} accumulates to a higher level than MP^{WT} and that MP^{S130A}fGFP is unable to transport itself from cell to cell are in line with this hypothesis. To study whether phosphorylation of the conserved S130 in the uninfected cells might be the trigger for tubule disassembly, S130 was replaced with an aspartic acid (D), which mimics a phosphorylated serine (Dean & Koshland, 1990). Unexpectedly, this abolished targeting to the plasma membrane, both in protoplasts (Fig. 3F) and epidermis cells (data not shown) and, as expected, cell-to-cell movement (Table 1). Furthermore, as observed for MP^{S130A}fGFP, cells expressing MP^{S130D}fGFP also seemed to accumulate more fluorescence than protoplasts expressing MP^{WT}fGFP (data not shown). Western blot analysis of transfected protoplasts confirmed that this corresponds to a higher amount of protein (Fig. 4, lanes 3 and 6). Furthermore, as for MP^{S130A}fYFP, incubation with BFA did not increase the expression level of MP^{S130D}fYFP in protoplasts (data not shown). Since MP^{S130D} does not target to the plasma membrane and does not form tubules, it does not provide any information on the role of phosphorylation in disassembly of the tubules, although it does suggest that S130 must be non-phosphorylated for targeting of MP to plasmodesmata. The observation that both MP^{S130A} and MP^{S130D} were more stable than MP^{WT} indicates that it is unlikely that phosphorylation of S130 is the trigger for degradation of MP.

DISCUSSION

Phenotypic analysis of a variety of mutant MPs in both epidermal leaf cells and protoplasts has shown that tubule formation by CPMV MP is the result of a sequence of different steps involving different domains in the MP. At first the MP is targeted to the plasma membrane, a process that likely requires di- or multimerization of the MP. At the plasma membrane MP accumulates in punctate spots, from which the tubules are probably assembled to grow into the neighbouring cell. The results with mutants MP^{S130A} and MP^{S130D} indicate that subsequently the tubules are disassembled in that cell to release virus particles that initiate a new infection.

Several of the different steps in tubule formation were identified using C-terminal deletion mutants. The observation that $MP^{1–251}$ mainly accumulated in the plasma membrane (Fig. 2D) and only occasionally in peripheral punctate structures indicates that MP accumulates in the plasma membrane before accumulating in punctate structures. Results obtained with MP^{S130A} and $MP^{1–276}$, which accumulate in the plasma membrane in addition to peripheral punctate spots, further strengthen this hypothesis. On the other hand we cannot rule out that, though unlikely, deletion of amino acids 252–276 exposes a membrane-interacting domain. The results obtained with the C-terminal deletion mutants also point towards a role for a region between amino acids 228–251 in targeting MP to the plasma membrane (Fig. 2E) and for a region between amino acids 252–276 in accumulation in punctate structures (Fig. 2D). However, the finding that mutations in several other regions of the MP also have an effect on targeting to the plasma membrane (Bertens *et al.*, 2000) suggests that this process is not brought about by a simple linear sequence. Possibly several regions will have to come together in a three-dimensional fold. This is probably also true for accumulation in punctate structures since a GFP variant with a plasma membrane anchor fused to amino acids 230–295 of the MP did not accumulate in peripheral punctate structures (data not shown).

Bertens *et al.* (2003) have determined that deletion of the C-terminal 44 amino acids (299–342) did not disturb tubule formation but that deletion of the last 50 amino acids (293–342) strongly inhibited tubule formation. Our experiments showed that $MP^{1–295}$ was able to accumulate in peripheral punctate structures and form tubules (Fig. 2B), although tubule formation was less efficient compared to MP^{WT}. Together these data indicate that amino acids 293–298 play a pivotal role in assembly of the tubule, possibly in multimerization of MP or interaction with a host factor required for tubule formation. Analysis of C-terminal deletion mutants of the MP of *Cauliflower mosaic virus* (CaMV), which forms tubules on protoplasts (Kasteel *et al.*, 1996) and in plant tissue (Bassi *et al.*, 1974; Linstead *et al.*, 1988), revealed that the 27 C-terminal amino acids are not required for tubule formation and the 77 C-terminal amino acids are not required for targeting to peripheral punctate spots (Huang *et al.*, 2000). Thus the organization of the domains for tubule formation and targeting to peripheral punctate structures in the C terminus of CaMV MP is very similar to that of CPMV MP, suggesting that CaMV MP uses the same steps in tubule formation. However, a CaMV MP mutant that accumulates uniformly in the plasma membrane has not been described yet.

The data obtained with $MP^{\Delta1–4}$fGFP suggest that the N terminus of MP is essential for targeting MP to the plasma membrane. The N terminus of CaMV MP, however, is not important for targeting to peripheral punctate structures, although it is important for tubule formation (Huang *et al.*, 2000; Thomas & Maule, 1999), indicating that the N termini of CPMV and CaMV play different roles in targeting of MP. Possibly a domain similar in function to the N terminus of CPMV MP is present in another part of CaMV MP.

In the presence of MP^{WT} some cytoplasmic mutants accumulate in peripheral punctate structures and are incorporated in tubules, suggesting that di- or multimerization of MP precedes targeting to the plasma membrane. The observation that MP molecules can interact in a blot overlay assay (Carvalho *et al.*, 2003) strengthens this hypothesis. However, although an interaction clearly does occur between cytoplasmic mutant MP and MP^{WT} in protoplasts and between MP^{WT} molecules *in vitro*, we have no direct proof that MP–MP interaction is essential for targeting MP^{WT} to the plasma membrane. Alternatively, MP^{WT} is required to initiate or nucleate tubule formation, leading to

the recruitment of mutant MP, which is not able to initiate or nucleate tubule formation but can still be incorporated into growing tubules. These complementation experiments do show that the ability to interact with MP^{WT} molecules is intact in these cytoplasmic mutants, suggesting that amino acids 3–12, Y42, P92/V93 (AM2), S130 and S225 are not localized at the dimer and/or multimerization interface. Possibly these mutations interfere with the interaction of MP with the plasma membrane or a host factor in the plasma membrane. Mutants that could not be complemented by MP^{WT} might be specifically disturbed in the MP–MP interaction domain but we cannot rule out that introduction of these mutations disturbed the overall folding of the MP, although the mutations were very subtle. Although interaction between MP molecules has previously been implicated in tubule formation of *Alfalfa mosaic virus* (Sanchez-Navarro & Bol, 2001) and CaMV (Huang *et al.*, 2001; Thomas & Maule, 1999) this is the first report to indicate that MP–MP interaction is involved in targeting to the plasma membrane.

Using Western blot analysis it was shown that the levels of transiently expressed MP^{S130A}fYFP and MP^{S130D}fYFP are higher than the expression level of MP^{WT}fYFP (Fig. 4), indicating that mutation of the serine at position 130 had an effect on the stability of MP. The observation that BFA increased MP^{WT} protein levels in protoplasts but not accumulation of MP^{S130A}fYFP or MP^{S130D}fYFP suggests that the increased stability of MP^{S130A}fYFP or MP^{S130D}fYFP could be caused by evasion of a degradation pathway linked to the secretory pathway, by which MP^{WT} has been suggested to be degraded (Pouwels *et al.*, 2002b). MP degradation could be required for breakdown of tubules to release virus particles in neighbouring uninfected cells, a process which might be blocked for MP^{S130A} and therefore cause the cell-to-cell movement defect of MP^{S130A}. The observation that virus replication increases the stability of MP (Wellink *et al.*, 1993a) is in line with this hypothesis, since breakdown of the tubule preferably has to occur in neighbouring, uninfected cells. However, the observation that MP^{WT}fGFP is able to spread from cell to cell by itself and is then able to accumulate in spots in the cell wall of neighbouring uninfected cells (Fig. 2F) might suggest that the tubules are disassembled in functional MP subunits and would argue against a role for degradation of MP in releasing virus particles from tubules. The hypothesis that MP^{S130A} is blocked in disassembly of the tubule would explain why MP^{S130A} does not accumulate in spots in the walls of neighbouring, uninfected cells and instead accumulates in the plasma membrane of the infected epidermal cell (Fig. 3C, D). However, more experiments are needed to clarify the phenotypes of MP^{S130A}fYFP and MP^{S130D}fYFP and the possible role this amino acid plays in disassembly of tubules.

Except for MP^{S130A}, the ability of the mutant MPs to support cell-to-cell spread of the virus corresponded to their ability to form tubules on protoplasts and spots, which probably are short tubules, in the cell wall of epidermal leaf cells (Table 1). Interestingly, MP^{S225A} showed reduced tubule formation on protoplasts due to less efficient accumulation in peripheral punctate structures (the amount of foci and tubules was smaller and tubules were shorter compared to MP^{WT}) and showed a markedly reduced cell-to-cell spread in intact leaves, confirming the correlation between tubule formation and the ability to support cell-to-cell transport. Also, except for MP^{S130A}, the ability of the mutant MPs to transport themselves from cell to cell, as shown for wt MP (Gopinath *et al.*, 2003), corresponded to the mutant's ability to form tubules on protoplasts and spots in the cell wall of epidermal cells. This correlation supports the hypothesis that the spots in the cell wall of infected epidermal cells represent short tubular structures, which have to be disassembled in the neighbouring cells to release the virus particles and start a new infection.

The results presented in this paper allow us to refine the delineation of functional domains of CPMV MP and the model on tubule formation (Bertens *et al.*, 2000). First the MP is targeted to the plasma membrane, which seems to require di- or multimerization of MP. In the plasma membrane MP quickly accumulates in spots, possibly through an interaction with a host protein. Subsequently tubules, which probably grow out of the spots (Huang *et al.*, 2000; Pouwels *et al.*, 2002b), are formed through the plasmodesmata, thereby removing the desmotubule. The tubules grow into a neighbouring uninfected cell where they are disassembled, thereby releasing the virus particles that can start infecting the cell. Whether disassembly of the tubule is due to degradation of MP or disassembly into functional subunits remains to be clarified.

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