Molecular Characterisation of the Cowpea Mosaic Virus Movement Protein

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MOLECULAIR CHARACTERISATION OF THE COWPEA MOSAIC VIRUS MOVEMENT PROTEIN

Peter Bertens

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Stellingen

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Het is aannemelijk dat voor het transport van CPMV van cel naar cel nieuwe intercellulaire kanalen worden aangelegd en dat er geen gebruik wordt gemaakt van bestaande plasmodesmata.

De initiatie van buisvorming en de verdere verlenging van de buizen voor virustransport zijn twee van elkaar te onderscheiden processen.
dit proefschrift

Het RNA-bindende domein van het transporteiwit van het bloemkoolmozaïekvirus lijkt niet essentieel te zijn voor het transport van dit virus van cel naar cel.

De conclusie van Huang et al. (2000) dat brefeldine A interfereert bij de vorming van buizen die nodig zijn voor het transport van het bloemkoolmozaïekvirus, is gebaseerd op een te laag aantal getelde protoplasten en daarom niet overtuigend.
*Huang et al. (2000). Virology **271**, 58-64*

Het is niet goed om de oorsprong van plantenvirussen die in Nederland niet van nature voorkomen te verhullen door ze perse een volledig Nederlandse naam te willen geven. De naam 'cowpea mozaïekvirus' verdient verre de voorkeur boven 'koebonenmozaïekvirus'.
Lijst van officiële namen van Nederlandse planten-virussen: http://www.minlnv.nl/pd/

Men moet erop bedacht zijn dat insectenvirussen als Flock House Virus (FHV) zich kunnen verspreiden in transgene planten die transporteiwitten van plantenvirussen tot expressie brengen, hetgeen aanleiding kan geven tot het ontstaan van nieuwe virusvariëteiten.
American Society of Virology Meeting 2000, abstract P9-4, pp. 152

Het gebruik van pesticide-resistente landbouwgewassen leidt niet tot een afname maar juist tot een toename van het aantal weidevogels.
*Watkinson et al. (2000). Science **289**, 1554-1557*

Het stimuleren van het biotechnologisch onderzoek en van het starten van biotechnologische ondernemingen met speciale fondsen als het Actieplan Life Sciences zal alleen vruchten afwerpen als ook het algemene bedrijfsklimaat voor biotechnologische bedrijven wordt verbeterd.

Carpoolen kan worden gestimuleerd door auto's met 3 of meer inzittenden vrij te stellen van toekomstige spitstoeslagen en vrij toegang te geven tot betaalstroken.

Het aantal door vogelende biologen bezochte congressen is evenredig met het aantal door hem of haar gespotte vogels.

CONTENTS

OUTLINE OF THIS THESIS

Plants are susceptible to many infectious diseases caused by a diversity of pathogens like nematodes, fungi, bacteria and viruses. Among these different pathogens plant viruses are unique as they are not cellular organisms, but consist of small nucleoprotein particles that use cellular machineries of the host cell for their replication. A most important step in the life cycle of plant viruses is the spread of the virus from an infected cell to neighbouring, uninfected cells, a process known as cell-to-cell movement. Plants have in their thick rigid cell walls small channels connecting neighbouring cells, so-called plasmodesmata, which serve as gates for plant viruses to move from cell-to-cell. The diameter of plasmodesmata normally is too small to allow free passage of the rather large virus particles or viral nucleic acids, but plant viruses code for specific movement proteins (MP) that are involved in modification of the plasmodesmal structure and enable cell-to-cell movement of viruses. The research described in this thesis was part of a programme entitled "The role of plasmodesmata in virus transport and cell-cell communication", financed by a grant of the Council for Earth and Life Sciences (ALW) of the Netherlands Organisation for Scientific Research (NWO) in 1995. The subsidy provided financial support of three PhD research projects which were executed simultaneously. The programme was coordinated by Prof. dr. R.W. Goldbach, Laboratory of Virology, Wageningen University. One project, executed at the Institute of Molecular Plant Sciences, Leiden University (PhD student L. Jongejan, supervisor Prof. dr. J.F. Bol) aimed at the identification of host plant (*Nicotiana benthamiana*) proteins, which interact with the movement and coat proteins of two distinct viruses, alfalfa mosaic virus (AMV) and cowpea mosaic virus (CPMV) by a two-hybrid analysis. The second project (PhD student N.N. van der Wel, Laboratory of Virology, Wageningen University, supervisor Prof. dr. R.W. Goldbach) dealt with the *in situ* analysis of the movement proteins of AMV and CPMV in their interactions with host proteins and plasmodesmata. The third project, of which the results are described in this thesis, aimed at the identification and characterisation of functional domains of the CPMV movement protein.

In **chapter 1** we present a review of the different forms of movement proteins of plant viruses and the mechanisms used for virus spread in the plant. The viral MPs have been categorised in different classes and the properties of one MP of each class will be described in detail.

The goal of the research described in this thesis has been to gain insight into the mechanism of cell-to-cell movement of cowpea mosaic virus (CPMV). CPMV is a small plant virus consisting of icosahedral particles with a diameter of 28 nm (Figure 1). The genome of the virus consists of two single-stranded RNA molecules of positive polarity, RNA1 and RNA2, each encapsidated in a separate particle. The two RNA molecules both contain a small protein (VPg) at their 5' end and have a poly(A) tail at their 3' end. Both RNAs are translated into large polyproteins, which are subsequently cleaved by an RNA1-encoded proteinase into 15 intermediate and final cleavage products (Figure 1). For an infection of plants, both RNA1 and RNA2 are required. Genetic analysis of the CPMV genome has revealed that proteins coded by RNA1 are involved in viral RNA replication. RNA2 codes for four mature proteins: two capsid proteins (LCP and SCP) and two C-terminally overlapping 58K and 48K proteins (Figure 1). Mutational analysis has indicated that the 58K protein is essential for replication of RNA2 and that the 48K protein is the viral MP, which together with the capsid proteins (CPs), is required for cell-to-cell spread of the virus. In tissue of CPMV-infected plants, tubular structures that penetrate the cell wall through modified plasmodesmata can be observed. The tubules contain virus particles, suggesting that virus particles move from cell-to-cell through these tubules (Figure 2). The 48K protein is the main structural component of the tubule.

Similar structures were observed in protoplasts inoculated with CPMV and in protoplasts transiently expressing the MP alone, where they extend into the incubation medium and are enveloped by the plasma membrane.

In **chapter 2** efforts are described to identify different functional domains of the MP by alanine-scanning mutagenesis. The results of that analysis support previous notions that the MP can be broadly divided in two areas. The N-terminal and central regions of the MP are necessary for tubule formation, while the C-terminus has a different role in cell-to-cell movement.

This role is further analysed in chapter 3. First, the C-terminal border of the tubule-forming domain was determined more precisely by the analysis of several deletion mutants and of hybrid viruses that coded for a MP in which the tubule forming domain was exchanged for the corresponding domain of a MP from a related virus. In addition, evidence was obtained that support the idea that the C-terminus of the MP is located inside the tubular structure, and is involved in an interaction with virus particles.

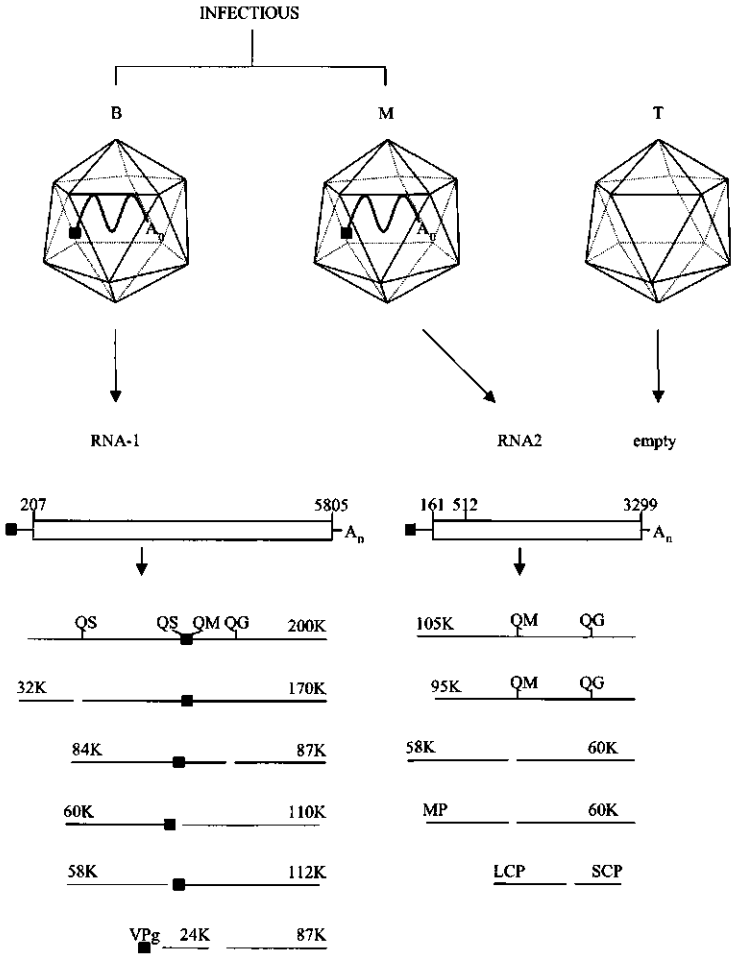

Figure 1: Genomic organisation and expression of the CPMV RNAs. CPMV has icosahedral particles with a diameter of 28 nm. Three different types of particles exist that differ in content. Bottom (B) and middle (M) particles each contain a segment of the single-stranded RNA genome (RNA1 and RNA2, respectively). Top (T) particles are empty. Both RNA1 and RNA2 are necessary for infections of plants.
RNA1 and RNA2 both possess a small protein, VPg, at their 5' ends and a poly(A) tail at their 3' ends. Open reading frames of RNA1 and RNA2 are shown as a bar. Nucleotide positions of start and stop codons are indicated. The synthesized proteins are indicated by black lines. QG, QM and QS cleavage sites are indicated in the 200K, 105K and 95K polyproteins. Abbreviations: MP, movement protein; LCP, large coat protein; SCP, small coat protein.

The development of reporter genes, like GFP, has been of great importance for the study of virus movement as this has made it possible to study the localisation and translocation of GFP-tagged viruses and of viral proteins fused to GFP in time.

Chapter 4 describes studies on the intracellular localisation and cell-to-cell spread of CPMV-derivatives coding for MP:GFP fusion proteins in plants and in protoplasts. Mutations in different parts of the MP indicated domains involved in targeting the MP to the cell membrane and plasmodesmata and the initiation and elongation of tubule formation.

Finally, in **chapter 5**, we discuss how the results have increased our insight in the movement of CPMV.

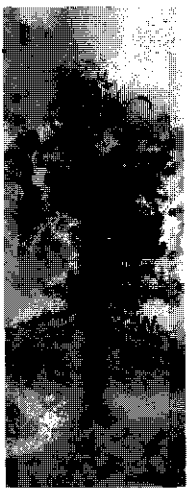

Figure 2: Electron micrograph of a tubular structure induced by CPMV in infected tissue. Arrows indicate the position of virus particles. CW, cell wall.

Chapter 1

PLANT VIRUS MOVEMENT PROTEINS

Abstract

One of the crucial steps in a viral infection of plants is the spread of the virus from an infected cell to a neighbouring, uninfected cell. Intensive research over the past decades has shown that this process, known as cell-to-cell movement, is controlled by a class of specialised viral proteins called movement proteins (MPs), that facilitate the transport of viral genomes through plasmodesmata, small channels that interconnect adjacent plant cells. In this review, we will give an overview of plant virus cell-to-cell movement and focus on the roles of the viral MPs in this process. MPs have been classified according to their mechanism of action and each mechanism will be illustrated by describing the properties of one MP in detail. The review will end with a short description of the phenomenon of *trans*-complementation and some remarks on the idea that plant viruses use a host mechanism for macromolecular transport through plasmodesmata for their own purposes.

Peter Bertens, Joan Wellink and Ab van Kammen
Submitted for publication

Introduction

Viruses are among the smallest of the pathogens known to infect plants, but their effects can be tremendous. Currently, more than 900 different plant viruses have been characterised and are classified in approximately 67 genera (Pringle, 1999). All these viruses greatly vary in shape and genome properties.

For successful infection of a plant by a virus, it is necessary that the virus first enters the host cells and replicates, and subsequently spreads throughout the entire plant to establish systemic infection. Plant viruses enter their hosts through wounds inflicted by an animal or fungal vector, or by mechanical damage of the epidermis. This results in an initial infection of one or a few cells of the host plant in which the virus replicates. The subsequent process of spreading through the plant can be divided into two phases. In first instance the virus moves to neighbouring, uninfected cells, a process known as short-distance transport or cell-to-cell transport. In that way, the infection can reach the vascular system and, if the phloem sap then takes up the virus, it will be transported to roots and higher leaves (long-distance transport). In these tissues, the virus may penetrate from the phloem into mesophyll cells and spread again further via cell-to-cell transport. Whereas this infection pathway applies to many plant viruses, several groups of plant viruses only infect a limited number of tissues or do not produce a systemic infection. For example, infection by certain gemini- and luteoviruses remains restricted to the phloem (e.g. Horns and Jeschke, 1991; Mayo and Ziegler-Graff, 1996).

Plant cells are surrounded by a rigid cell wall, that functions as a physical supporting structure and as a barrier against abiotic and biotic stress like pathogens. Viruses cannot simply pass the cell wall, but use different strategies to overcome this barrier. They exploit the plasmodesmata, natural connections between neighbouring plant cells, to move from cell-to-cell. Although this has already been postulated more than 50 years ago, clues about the mechanisms used by viruses to move from cell-to-cell have only emerged in the last 10-15 years.

In this review, we will present an overview of the role of a special class of viral proteins, referred to as movement proteins (MPs), in plant virus cell-to-cell movement (for other reviews on this subject see Carrington et al., 1996; Lazarowitz, 1999; Lazarowitz and Beachy, 1999; Lucas and Wolf, 1999 and McLean et al., 1997). We shall discuss the viruses that seem to spread as virus particles through tubular structures and the viruses that move as nucleoprotein complexes differing from virions. Given these two ways of virus movement, the MPs can be classified in several groups. These groups will be described in detail. At the end of the review, the remarkable phenomenon of *trans*-complementation of viral cell-to-cell movement is described and the idea that viruses

exploit a system for the transport of macromolecules through plasmodesmata developed by plants is elucidated. In the next section, first the structure and function of plasmodesmata is described, as this information is needed to fully understand the complexity of virus movement.

Structure of plasmodesmata

Although the occurrence of plasmodesmata in plants has been studied since several decades, little is still known about the molecular organisation of plasmodesmata and only recently some proteins have been described that seem to be characteristic for plasmodesmata (e.g. Blackman et al., 1999). Ultrastructural analysis of plant tissues has shown that there are several forms of plasmodesmata: primary plasmodesmata, which are formed during cytokinesis (reviewed in Lucas et al., 1993; Ding, 1997; Crawford and Zambryski, 1999), and secondary plasmodesmata that are formed later during development through existing cell walls and are involved in the expansion of the cytoplasmic continuum (Van der Schoot and Rinne, 1999). The cytoplasmic continuum refers to the combined cytoplasm of all cells interconnected by plasmodesmata, which enables communication through the plant. Not every cell in the mature plant is part of this cytoplasmic continuum. Down-regulation of the number of plasmodesmata during development leads to the formation of temporally or continuously isolated groups of cells. The stomata in the epidermis are examples of cells that have no connections through plasmodesmata with their neighbouring cells (Wille and Lucas, 1984).

In general, plasmodesmata are narrow, plasma membrane-lined channels, 20-30 nm in diameter, which cross the plant cell wall (Figure 1). Some plasmodesmata consist of only one channel (linear or simple plasmodesmata) whereas others consist of a network of channels (branched plasmodesmata) (Itaya et al., 1998). Both linear and branched plasmodesmata have a similar structure. The centre of the channel is composed of a stretch of appressed endoplasmic reticulum (ER), the desmotubule. The desmotubule and the plasma membrane are probably connected by protein molecules, in a way that the cytoplasmic sleeve, i.e. the space between plasma membrane and desmotubule, is subdivided into smaller microchannels, each with a diameter of 1.5-2.0 nm. Actin (White et al., 1994) and myosin (Reichelt et al., 1999; Redford and White, 1998) might be structural components of these microchannels. Small metabolites, like phytohormones, lipids and other small molecules, can diffuse from cell-to-cell via these microchannels, but larger molecules like proteins and RNA molecules can not pass plasmodesmata freely. In order to allow translocation of these proteins across plasmodesmata, the molecular size exclusion limit (SEL) has to increase. Originally, it was thought that plasmodesmata are quite static structures with a SEL of 0.8-1.0 kDa (Terry and Robards, 1987). However, recent studies show that the SEL of

plasmodesmata is very dynamic and can be influenced by environmental (Cleland et al., 1994; Schulz, 1995) and developmental signals (Duckett et al., 1994; Oparka et al., 1999). Furthermore, plasmodesmata may have tissue-specific features (Waigmann and Zambryski, 1995; Kempers and Van Bel, 1997). During development and maturation, leaves undergo a transition from 'sink tissue' (nutrients importing region) to 'source tissue' (nutrients exporting region) (Turgeon, 1989). In sink tissues of tobacco plants, the SEL of plasmodesmata is at least 50 kDa (Oparka et al., 1999), much larger than initially thought. In leaves undergoing the sink-to-source transition, the SEL is gradually decreasing to a value around 1 kDa. This decrease seems to be correlated with a structural change from linear to branched plasmodesmata (Oparka et al., 1999).

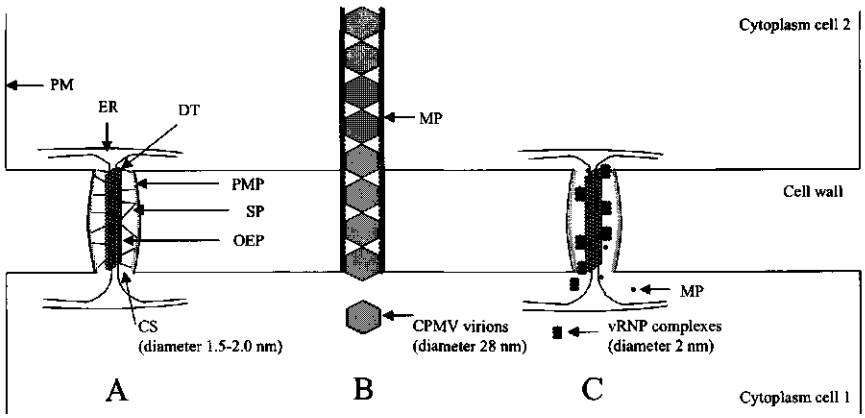

Figure 1: Schematic representation of the structure of a linear plasmodesma, several plant viruses and naked viral RNA. Abbreviations: CS, cytoplasmic sleeve; DT, desmotubule; ER, endoplasmic reticulum; OEP, outer ER proteins; PM, plasma membrane; PMP, plasma membrane proteins; SP, spike proteins; vRNP, viral nucleoprotein complex. The diameter of the transport channel, 2 nm, is indicated as a short bar. The diameter of the naked viral RNA is according to Citovsky et al. (1992).

Viruses and plasmodesmata

Both viral particles and naked viral RNA are too large to spread through plasmodesmata by diffusion (Figure 1). Virus particles considerably differ in size and shape (Matthews, 1991). Small plant viruses, like the isometric nano- and comoviruses, have a diameter between 17 and 30 nm. Larger viruses can have rod-shaped particles (length between 65-350 nm, width between 15-25 nm), bacilliform particles (30-500 nm in length, 18-35 nm in width), or rhabdo-shaped particles (95-1350 nm in length, width of 3-8 nm). The largest plant viruses known have filamentous particles, with lengths of up to 2000 nm and a width between 3-20 nm. Naked viral RNA, that has a coiled structure, still has

a diameter of around 10 nm (Citovsky et al., 1992). Even in 'sink' tissue, which has plasmodesmata with a SEL of over 50 kDa, viruses cannot simply diffuse to neighbouring cells (Oparka et al., 1999). So, how do plant viruses overcome this problem?

In the 1960's, ultrastructural investigations of infected plant tissues revealed the presence of tubular structures filled with virus particles which were located inside plasmodesmata (e.g. Walkey and Webb, 1968, Davison, 1969). It was suggested that these tubular structures, found in plasmodesmata of infected cells, could be a way of intercellular viral transport (Kitajima and Lauritis, 1969). At that time, functional studies on these tubular structures were not conducted. Progress in understanding viral movement was made by the use of genetic and molecular biological techniques since the end of the 1970's. Analysis of temperature-sensitive (*ts*) isolates of tobacco mosaic virus (TMV) identified a protein that specifically is involved in cell-to-cell movement of TMV (Leonard and Zaitlin, 1982; Meshi et al., 1987; Deom et al., 1987). This was the first example of a viral MP, which was later found to act by unfolding the viral RNA into a linear molecule that is small enough to pass plasmodesmata. Since then, MPs have been identified in plant viruses of most genera and more details of their mechanism of action are being elucidated.

A further important development for the study of virus movement was the introduction of marker proteins. Initially *Escherichia coli*-derived β-glucuronidase (GUS) was widely used as a marker gene, but nowadays the jellyfish green fluorescent protein (GFP) has taken over the role of most popular marker gene to study the localisation of viruses and viral proteins in tissues and cells. Without fixation of the infected tissue GFP fluorescence can be located in individual cells using high resolution confocal laser scanning microscopy (Oparka et al., 1997b).

Classification of MPs

As mentioned before, plant viruses show a large variation in genome composition, number of genome segments and particles and particle morphology (Matthews, 1991). This diversity cannot be found for their cell-to-cell movement, since plant viruses seem to use only two basic mechanisms. However, MPs of viruses belonging to different families show surprisingly little homology in amino acid sequence. There appears to be no correlation between the form of the viral particle, and the encoded MP and neither between the type of viral genome and the encoded MP. Based on the amino acid sequence homology detectable between viral MPs and the mechanism of action of the MPs, one can divide viral MPs into eight classes. Class I consists of MPs that form tubular structures within plasmodesmata. These tubular structures contain virus-like

particles that spread in this way to neighbouring cells. As a typical example of this group, we will describe the characteristics of the cowpea mosaic virus (CPMV) MP in more detail. A second class consists of viruses that spread cell-to-cell as a ribonucleoprotein (RNP) complex through plasmodesmata that show no obvious morphological modifications. The TMV MP will be described as an example of this class of viral MPs. The third class of MPs is comprised of several virus groups that have not one MP, but a cluster of three MPs, encoded by the so-called triple gene block (TGB). A fourth class is formed by MPs that have characteristics of both classes I and II MPs (we will summarise alfalfa mosaic virus (AMV) cell-to-cell movement). Cell-to-cell movement of potyviruses (class V), carmo- and necroviruses (class VI), closteroviruses (VII) and poleroviruses (class VIII) are described shortly. At the end of this review, we will make some remarks concerning the homologies that have been found between viral MPs and between MPs and plant proteins. Furthermore, we will describe the phenomenon that a spread of a movement-defective virus can be complemented by the actions of a different MP and discuss the possibility that plant viruses use a host-derived system for plasmodesmal trafficking.

Tubule-guided movement

Viruses of various groups use tubular structures formed in extensively modified plasmodesmata to move from cell-to-cell. These structures were first noted more than 25 years ago, in plant tissues infected with como- (Van der Scheer and Groenewegen, 1971; Kim and Fulton, 1971), nepo- (Walkey and Webb, 1968; Davison, 1969), faba- (Hull and Plaskitt, 1974), fiji- (Gerola and Bassi, 1966; Jones and Roberts, 1977), sequi- (Murant et al., 1975) and caulimoviruses (Kitajima and Lauritis, 1969; Kitajima et al., 1969) (see Table 1). As this type of cell-to-cell movement has been studied most extensively for CPMV, we will discuss the model of tubule-guided movement of CPMV in detail.

Involvement of 48K in cell-to-cell movement

CPMV is the type member of the comoviruses, a group of plant viruses that have icosahedral particles with a diameter of 28 nm. There are three kinds of viral particles; two of which each contain a single stranded RNA molecule of positive polarity and the third type of particle is empty. Both RNA1 and RNA2 are translated into large polyproteins, which are cleaved by a virally encoded proteinase (for a review see

Goldbach and Wellink, 1996). RNA1 codes for the proteins involved in replication of the viral RNAs. RNA2 codes for two coat proteins (CPs), and C-coterminal 48K and 58K proteins. Genetic evidence for the presence of a MP was obtained by Wellink and Van Kammen (1987), who showed that both the CPs and the 58K/48K proteins are involved in cell-to-cell movement. Since the 48K but not the 58K product was detected in the membrane fraction of infected leaves, the 48K protein is referred to as the CPMV MP (Rezelman et al., 1989). Interestingly, 48K and the CPs do not need to be expressed *in cis*, since a tripartite virus that contained 48K and CP genes on different RNA molecules, was fully infectious (Verver et al., 1998). In cells of plant tissue infected with CPMV, plasmodesmata contain long tubular structures that extend from the entry of the plasmodesmata in one cell into the cytoplasm of the neighbouring cell (Van Lent et al., 1990). These tubular structures have a diameter of ~30 nm and contain single rows of virus particles. The tubule-containing plasmodesmata have a diameter around 34 nm, which is much larger than the diameter of 2 nm of unmodified plasmodesmata. A desmotubule is not apparent in the tubule-containing plasmodesmata that seem to be drastically modified. Using antibodies against the 48K protein it was shown by immunogold labelling that these tubules contain 48K protein, suggesting that the role of the 48K protein in virus movement might be the formation of the tubular structure (Van Lent et al., 1990). A typical feature is that tubular structures filled with virus-like particles, similar to those found in infected tissue, can also be formed on protoplasts inoculated with CPMV (Van Lent et al., 1991). In protoplasts, these tubular structures originate close to the plasma membrane and can protrude up to 20 µm into the incubation medium while being surrounded by plasma membrane. It gives the impression that the tubules in protoplasts push themselves outwards of the cell. Mutational analysis of the CPMV genome has shown that the 48K protein is the only viral protein required for tubule formation in protoplasts and that the viral capsid proteins or viral particles are not needed (Kasteel et al., 1993; Wellink et al., 1993). The protoplast studies further indicate that intact plasmodesmata are not essential for the assembly of tubular structures. In this context it is interesting that tubular structures are also formed in protoplasts from nonhost plants of CPMV upon inoculation with CPMV RNAs. Apparently tubule formation by the MP does not require a very specific host determinant (Wellink et al., 1993). Even more remarkable is that tubular structures can also be formed upon expression of the MP in insect cells, in which the tubules also protrude outwards into the culture medium. Therefore, if host proteins are involved in tubule formation, these should be of a conserved nature among animals and plants (Kasteel et al., 1996). In tubule-enriched fractions isolated by differential centrifugation of CPMV-infected protoplasts, the 48K protein and the two CPs are the major specific proteins present, as analysed by SDS-PAGE (Kasteel et al., 1997). So far, it remains to be

established whether, and if so which, host components are involved in the process of targeting of MP to the outer membrane where tubule formation occurs, or in other steps in the cell-to-cell transport of CPMV.

Mutational analysis has indicated that the CPMV MP has various functional domains. The C-terminus is probably involved in an interaction between MP and virus particles (Lekkerkerker et al., 1996) and has a role in proteolytic cleavage of the MP from a polyprotein precursor (Gopinath et al., 2000). Tubular structures composed of 48K containing an 18 amino-acid deletion in its C-terminus do not contain virus particles, although these are present in the cytoplasm of inoculated cells. This mutant virus is not infectious, showing that CPMV exclusively moves in a virion form from cell-to-cell through tubular structures. The remaining part of the MP is essential for tubule formation (Lekkerkerker et al., 1996). Within this tubule-forming domain different regions have been identified that are involved in the targeting of 48K to the cell membrane (Bertens et al., 2000).

A model for tubule-guided cell-to-cell movement

The experimental data described above can be summarised in a model (Figure 2A). CPMV replication and translation occurs on membranous vesicles (Goldbach and Wellink, 1996, Carette et al., 2000). MP and/or 58K was found to be associated with these structures. After translation, 48K protein is targeted to plasmodesmata by an yet unknown mechanism. The tubular structures are composed mainly or even entirely of 48K molecules, suggesting that 48K has the ability to self-aggregate. The aggregation process probably occurs near plasmodesmata and may be initiated by an earlier interaction between 48K and a membrane protein. Since tubular structures can be formed in cells lacking plasmodesmata (protoplasts and insect cells), plasmodesmal components do not seem to be vital for tubule formation per se. However, plasmodesmal proteins may have a function in confining tubule formation to plasmodesmata. The formation of tubules will require interactions between 48K and plasmodesmal components, as the result of tubule formation is a drastic modification of the plasmodesmatal structure that includes the removal of the desmotubule. The energy required for such a process might be generated by interactions of 48K with host components or, alternatively, may be released during the assembly of 48K into tubular structures. It is still obscure how viral particles are translocated across the modified plasmodesmata. Most likely is a model in which the tubule containing virus particles passes through a plasmodesma and deposits virus particles in a neighbouring cell. In the neighbouring cell the tubular structure is degraded, releasing the viral particles. This degradation might be an active process, whether or not requiring a host factor.

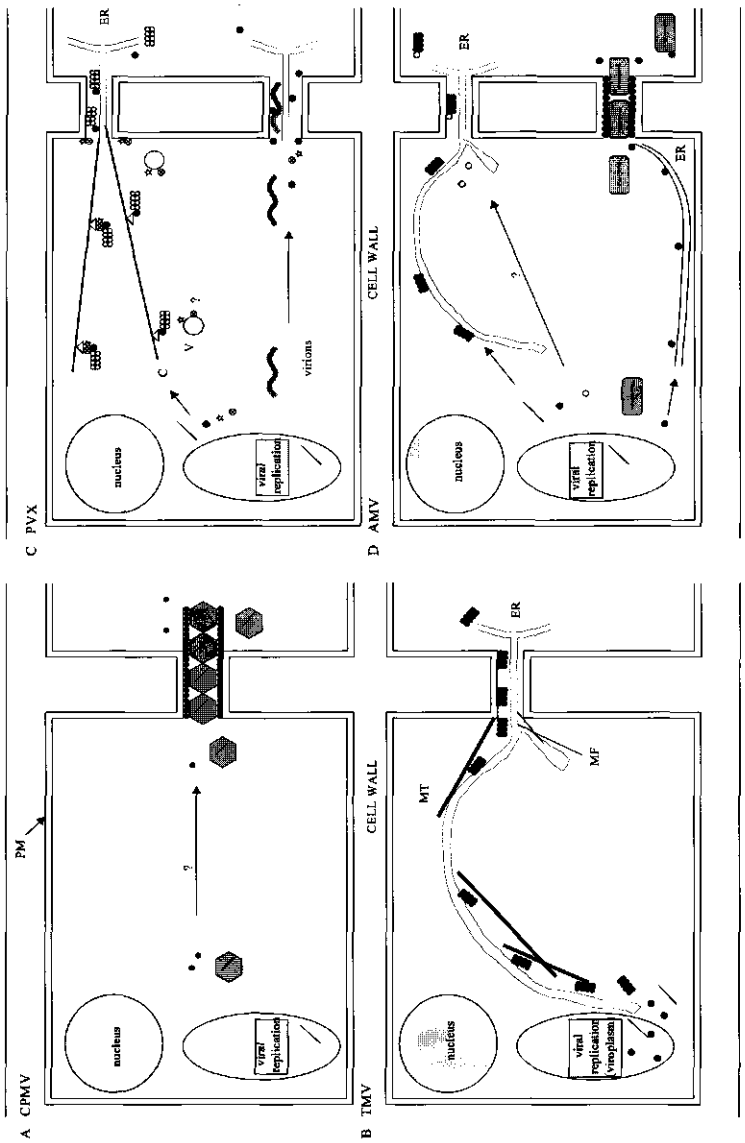

Figure 2. Models of the cell-to-cell movement mechanisms of CPMV (A), TMV (B), PVX (C) and AMV (D). In all figures, MP is shown as a black circle. In A, C, and D virus particles are shown. Single CPs are indicated with an open circle, and viral RNA with a short bar. In (C), TGBp2 (closed cross) and TGBp3 (star) are also indicated. The triangle in (C) represents a putative host complex. Abbreviations: C, cytoskeleton; ER, endoplasmic reticulum; MF, microfilaments; MT, microtubules; PM, plasma membrane; V, vesicles.

An alternative view is that the tubule is a rigid structure embedded in the modified plasmodesma through which the viral particles flow. Since in CPMV-infected plants tubular structures devoid of virus particles have not been detected, a model in which virus particles are incorporated into the tubular structure during tubule formation is favourable over a model in which the virus particles enter a mature tubule.

Other viruses moving cell-to-cell through tubular structures

Tubular structures containing virus-like particles have also been found in plants infected with other comoviruses (Kim and Fulton, 1971; Shanks et al., 1989), nepo-, faba-, fiji-, sequi- and caulimoviruses, and in addition more recently in plants infected with tospo- (Kormelink et al., 1994) and badnaviruses (Cheng et al., 1998). This is a diverse group of plant viruses. Most of these viruses have nonenveloped icosahedral nucleoprotein particles, but tomato spotted wilt virus (TSWV), the type member of the tospoviruses, has enveloped spherical particles with a diameter of 80 nm and badnaviruses have bacilliform particles with a length up to 400 nm. Several types of viral genomes can be found among these viruses. Most have single stranded RNA genomes of positive polarity or ambisense polarity (tospoviruses), but badna- and caulimoviruses contain a double stranded DNA genome and fijiviruses a double stranded RNA genome.

Nepoviruses have isometric particles of up to 33 nm in diameter. They are closely related to comoviruses with regard to particle morphology, genome organisation and expression strategy (Mayo and Robinson, 1996), although their MPs seem not to be closely related (Mushegian, 1994). Cells infected with nepoviruses contain numerous tubular structures, many of which can be found in the cytoplasm (Roberts and Harrison, 1970; Walkey and Webb, 1970). By immunogold labelling, a protein encoded by the central region of RNA2 was found to be a component of the tubular structures and thus identified this protein as the nepoviral MP (Wieczorek and Sanfacon, 1993; Ritzenthaler et al., 1995). Analysis of chimeric RNA2 molecules consisting of part of either the RNA2 molecules of arabis mosaic virus (ArMV) and grapevine fanleaf virus (GFLV) suggested that the C-terminus of the GFLV MP is likely to be involved in an interaction with virus particles (Belin et al., 1999), similar to what has been proposed for CPMV.

The MPs of faba-, sequi- and fijiviruses are less well characterised. These three groups have isometric virus particles, with a diameter of 25 nm, 31 nm and 80 nm, respectively. The sole evidence available that these viruses spread in a comoviral fashion, is that tubular structures containing virus-like particles have been detected in plants infected with such viruses (Gerola and Bassi, 1966; Hull and Plaskit, 1974; Murant et al., 1975). Furthermore, the putative MP of fabaviruses has limited sequence homology to other tubule-forming MPs (Ikegami et al, 1998).

Caulimo- and badnaviruses are the two genera of plant viruses that contain a double stranded DNA genome (Franck et al., 1980; Medberry et al., 1990)). In tissues infected with cauliflower mosaic virus (CaMV), the type member of the caulimoviruses, tubular structures similar in appearance to those seen in CPMV-infected tissues have been observed (Conti et al., 1972). These tubules contained isometric virus particles (with a diameter of 50 nm) and can be immunolabeled with antisera raised against the product of the P1 gene (Linstead et al., 1989). This, and the observation that deletions in P1 have an effect on intercellular spread of CaMV (Thomas et al., 1993), identifies P1 as the CaMV MP. Several forms of P1 are detectable by Western blot analysis of infected tissue (Harker et al., 1987), but only one form, a 46 kDa protein, is found in tubular structures (Perbal et al., 1993). The structure of the CaMV tubules is unknown, although results of a mutational analysis suggested that the conserved central region of the MP has a structural role, and that the N- and C-terminal regions are exposed on the outer and inner faces of the tubule respectively (Thomas and Maule, 1995a). This suggestion is supported by recent studies that show that deletions in the C-terminal region of P1 do not affect tubule formation. However, deletions in the N-terminal region block tubule formation (Thomas and Maule, 1999). Co-expression studies with wild type and mutant MP indicated that two regions of the CaMV MP (amino acids 127-141 and 248-277) are involved in self-association (Thomas and Maule, 1999). Furthermore, it has been reported that the P1 protein has RNA-binding activity, a characteristic of MPs classified in other groups (Thomas and Maule, 1995b). This binding is rather weak compared to the RNA-binding characteristics of the MPs of TMV and red clover necrotic mosaic virus (RCNMV) (Citovsky et al., 1991). P1 binds RNA with a 10-fold greater affinity than ssDNA, again different from the TMV MP, which binds RNA and ssDNA with a similar affinity (Citovsky et al., 1990, 1991). Attempts to further specify this domain have so far been unsuccessful (Thomas and Maule, 1995b). It is still unclear whether the RNA-binding properties of P1 have any function in viral movement or that this property is an evolutionary remnant, functional in an ancestor of P1. It remains also possible that the RNA-binding properties have a role specifically in the 38 kDa translation product and not in the 46 kDa product.

Badnaviruses code for three proteins, the largest of which is a polyprotein that codes for several viral proteins including a proteinase and the CP (Medberry et al., 1990). The tubular structures found in badnavirus-infected cells contain bacilliform-shaped particles (Cheng et al., 1998). The badnaviral MP was identified by its sequence similarity to known MPs and found to be located in the N-terminal part of the polyprotein (Bouhida et al., 1993). Furthermore, mutations in this area have an effect on cell-to-cell movement but not on replication of the virus (Tzafrir et al., 1997). Antisera raised against the putative MP labelled the tubular structures observed in infected plant tissue

(Cheng et al., 1998), confirming that this region of the polyprotein indeed acts as the viral MP.

The spherical particles of TSWV have a diameter of 80-110 nm. In plasmodesmata of infected cells, tubular structures can be found with a diameter of 40-45 nm that contain non-enveloped, viral nucleocapsids (Storms et al., 1995; Kikkert et al., 1999). Tubular structures formed after infection of protoplasts with TSWV show a clear filamentous substructure (Storms et al., 1995). The tubules can be labelled with antisera raised against NS_M, the TSWV MP. Tubules are detected early and only transiently during viral infection, coinciding with the expression pattern of NS_M (Kormelink et al., 1994; Storms et al., 1995). NS_M also associates with viral nucleocapsids in the cytoplasm, suggesting that NS_M plays a role in targeting of nucleocapsids to the tubular structures. Recent results described by Soellick et al. (2000) suggest that the NSM protein can interact with TSWV N protein, the main component of the nucleocapsid, *in vitro*, and can bind single-stranded RNA in a sequence-nonspecific manner. Several other groups of plant viruses are also thought to spread from cell-to-cell as virus particles through tubular structures, but have been less fully investigated, and therefore these groups are indicated in italics in Table 1.Although the MPs of these various viruses all form tubular structures for cell-to-cell movement of virus particles, these proteins show little similarity to each other. A comparison of the amino acid sequences of the MPs involved in tubule-guided transport revealed the presence of only short stretches of conserved amino acids, concentrated in the central regions of these MPs (e.g. Thomas et al., 1995; Mushegian and Koonin, 1993). Surprisingly, there is no homology between the primary sequences of como- and nepoviral MPs and very little between nepoviral MPs (Mushegian, 1994). Different functional domains of caulimo- and comovirus MPs, and possibly also nepoviral MPs might be arranged in a similar way (Thomas et al., 1995; Belin et al., 1999). For these MPs it is thought that the central region has a structural role (tubule formation), while the C- and/or N-termini are exposed to the outside or inside of the tubule. There are indications that the C-termini of these MPs are involved in an interaction between MP and virus particles (Lekkerkerker et al. 1996; Belin et al., 1999). So, in conclusion one can remark that there is no high sequence identity between tubule-forming MPs at the amino acid level, but all these MPs may have a common tertiary structure.

Table 1: Overview of viruses coding for group I MPs

genus[a]	virus[b]	genome type	segments	particle morphology	CP requirement[c]	MP
Comovirus	CPMV	ssRNA	2	isometric	+	48K[1]
Nepovirus	GFLV	ssRNA	2	isometric	+?	P38[2]
Fabavirus	*PaMMV*	*ssRNA*	*2*	*isometric*	*+?*	*52K*[3]
Sequivirus	*PYFV*	*ssRNA*	*1*	*isometric*	*+?*	*N-terminus polyprotein?*[4]
Tospovirus	TSWV	ssRNA	3	spherical	+?	NS_M[5]
Fijivirus	FDV	dsRNA	10	isometric	+?	P7-1[6]
Badnavirus	CoYMV	dsDNA	1	bacilliform	+?	ORFIII[7]
Caulimovirus	CaMV	dsDNA	1	isometric	+	P1[8]
SoyCMV-like	*SoyCMV*	*dsDNA*	*1*	*isometric*	*?*	*ORFIb*[9]
CVMV-like	*CVMV*	*dsDNA*	*1*	*isometric*	*?*	*part of ORF-I*[10]
PVCV-like	*PVCV*	*dsDNA*	*1*	*isometric*	*?*	*ORF1*[11]
RTBV-like	*RTBV*	*dsDNA*	*1*	*isometric*	*?*	*N-terminus of P3*[12]

[a] Viruses that spread as virions or nucleocapsids (tospoviruses) through tubular structures formed by their MP in modified plasmodesmata. Groups shown in *italic* are putatively classified in this group due to the amino acid homology found between their MPs and other group I MPs or because tubular structures enclosing virus-like particles have been detected in modified plasmodesmata.

[b] CPMV, cowpea mosaic virus; GLFV, grapevine fanleaf virus; PaMMV, patchouldi mild mosaic virus; PYFV, parsnip yellow fleck virus; TSWV, tomato spotted wilt virus; CoYMV, Commelina yellow mottle virus; FDV, Fiji disease virus; CaMV, cauliflower mosaic virus; SoyCMV, soybean chlorotic mottle virus; CVMV, cassava vein mosaic virus; PVCV, petunia vein-clearing virus; RTBV, rice tungro bacilliform virus

[c] Involvement of the CP in cell-to-cell movement. Key: +, genetic evidence provided for a CP requirement; +?, no genetic evidence provided that CP is involved, but morphological data supporting a role for the CP in movement; ?, not known

Key references: 1, Van Lent et al. (1990); 2, Ritzenthaler et al. (1995); 3, Ikegami et al. (1998); 4, Turnbull-Ross et al. (1993); 5, Kormelink et al. (1994); 6, Isogai et al. (1998); 7, Tzafrir et al. (1997); 8, Perbal et al. (1993); 9, Hasegawa et al. (1989); 10, Calvert et al. (1995); 11, Richert-Poggeler et al. (1997); 12, Hay et al. (1991)

Movement as ribonucleoprotein complexes – TMV-like

TMV serves as a model for viruses that use a mechanism for cell-to-cell movement quite different from that of the tubule-forming viruses described before. Plasmodesmata of cells infected with TMV are not visibly modified (Shalla et al., 1982) and no structure can be observed that might be connected with virus movement. Almost 40 years ago, mutants of TMV were found which did not form mature virus particles, but still could move from cell-to-cell (Siegel et al., 1962). A few years later, TMV mutants were characterised that were temperature-sensitive (*ts*) in cell-to-cell movement whereas these mutants normally accumulated in protoplasts at non-permissive temperatures (Jokusch et al., 1968). Further characterisation of the cell-to-cell mechanism of TMV started 15-20

years ago with the molecular analysis of the genome of *ts* mutants and the conclusion that TMV contains a gene that mediates its cell-to-cell movement (Bosch and Jokusch, 1972; Nishiguchi et al., 1978).

Involvement of P30 in cell-to-cell movement

Comparison of the genome sequence of *ts* mutants with that of the wild-type strain of TMV showed differences within the P30 protein ORF (Leonard and Zaitlin, 1982; Zimmern and Hunter, 1983). Subsequently, these differences were shown to be associated with the *ts* cell-to-cell movement (Meshi et al., 1987) and shortly after that it was proven that the 30K (or P30) protein is the MP of TMV, when it was shown that transgenically expressed P30 is able to complement the *ts* phenotype of the movement mutants (Deom et al., 1987). By immunogold labelling of infected tissue, Tomenius et al. (1987) showed that P30 can be detected in plasmodesmata from 16 hours post inoculation (h.p.i.) onwards. Maximum amounts of P30 were found 24 h.p.i. after which there was a decrease in the amount of plasmodesmata labelled and the intensity of the label.

Since the plasmodesmata in TMV-infected tissue and in transgenic plants expressing the P30 are much too small to allow passage of TMV particles and do not have gross structural modifications, how then does P30 enable cell-to-cell movement of TMV? Wolf et al. (1989) showed, using micro-injection techniques, that the molecular size exclusion limit (SEL) of plasmodesmata was increased in P30-transgenic tobacco plants: in normal plants, plasmodesmata allow passage of molecules with a mass of up to 0.8 kDa, but the SEL in transgenic plants is increased to over 9.4 kDa. In TMV-infected plants this increase in SEL is a transient process and only occurs at the leading edge of infection (Oparka et al., 1997a). The transient increase in SEL corresponds with the expression pattern of P30 in infected plants (Joshi et al., 1983; Ooshika et al., 1984).

The enlargement of the plasmodesmal SEL leads to an expansion of the diameter of the plasmodesmal channel from 0.73 nm in non-transgenic plants to 2.4-3.1 nm in transgenic plants expressing P30 (Wolf et al., 1989; Waigmann et al., 1994). This enlargement is insufficient to allow cell-to-cell movement of TMV virions (which have a diameter of 18 nm) or naked RNA (that has a diameter of approximately 10 nm). The problem is overcome by the formation of complexes between viral RNA and P30. Citovsky et al. (1990) showed that P30 is able to bind and unfold both single-stranded RNA and DNA *in vitro*. This binding is strong, cooperative and sequence non-specific (Citovsky et al., 1990). Each MP molecule can bind a stretch of 4-7 nucleotides on the RNA molecule (Citovsky et al., 1990). The diameter of extended RNA-MP complexes is in the order of 2 nm, allowing transport through the enlarged plasmodesmata.

The intracellular movement of the TMV MP from its site of synthesis to plasmodesmata was unravelled using a TMV derivative coding for a fusion protein of P30 and GFP able to infect whole plants (Heinlein et al., 1995). The P30-GFP fusion product was associated with elements of the cytoskeleton and endoplasmic reticulum (reviewed in Reichel et al., 1999). In all infected cells, P30-GFP fluorescence was detectable as punctate spots in the cell wall, associated with plasmodesmata (Oparka et al., 1997a). Micro-injection experiments of P30 and fluorescently labeled dextrans demonstrated that P30 is capable of moving itself from cell-to-cell rather rapidly and makes likely that P30 uses a host pathway for plasmodesmatal transport of macromolecules (Waigmann et al., 1994). Fusion proteins consisting of P30 and GUS were also able to traffic from cell-to-cell, whereas GUS alone did not move (Waigmann and Zambryski, 1995). This could be explained by assuming that P30 contains a specific sequence responsible for plasmodesmatal targeting (a plasmodesmata targeting signal, PTS) (reviewed in Lucas et al., 1993; Wolf and Lucas, 1994; Ding et al. 1997). The putative targeting signal has not yet been characterised, however.

In protoplasts inoculated with recombinant TMV encoding GFP-tagged P30, early during viral infection, P30-GFP fluorescence accumulated in small punctate bodies around the nucleus interconnected by a network of fluorescent filaments (Heinlein et al., 1998; Más and Beachy, 1998; Reichel and Beachy, 1998). These bodies were formed out of cortical ER and grew in size until they reached their maximum size 20 hpi (Heinlein et al., 1998). Near the periphery of the cell, small fluorescent punctae were observed that closely aligned to microtubuli and might be related to cell wall adhesion sites, sites where microtubules attach to the cell wall (Heinlein et al., 1998). In a later stage of the infection fluorescence is detectable in plasma membrane protrusions, reminiscent of the tubular structures that are formed during infection with comoviruses (Heinlein et al., 1998). The exact composition or function of these protrusions is unknown. Also, during these late stages of protoplast infections, P30-GFP fluorescence was colocalised with microtubules close to the plasma membrane (Heinlein et al., 1995, 1998; Más and Beachy, 1998). P30 also colocalised to some extent with actin and has been shown to directly bind to actin *in vitro* (McLean et al., 1995).

The increase in plasmodesmal permeability following TMV infection directly affects communication between plant cells and may have large effects on the physiology of the host plant. To minimise these effects, TMV may have developed mechanisms to regulate P30 activity, including the precise timing of P30 expression and its rapid inactivation after translocation of TMV genomes. P30 may be inactivated via phosphorylation and/or proteolytic processing. Citovksy et al. (1993) showed that P30 is phosporylated in tobacco plants by a developmentally regulated protein kinase that is associated with the cell wall. Mutagenesis of the phosphorylated amino acids affects

P30's ability to increase plasmodesmal permeability, suggesting that phosphorylation acts in the down-regulation of this protein (Citovsky, 1999, Chen et al., 2000). However a different mechanism may be used to inactivate P30 in *Arabidopsis thaliana*, where P30 is not phosphorylated, but proteolytically cleaved at its N-terminus (Hughes et al., 1995). The inability to complement the movement of a TMV P30 frameshift mutant in transgenic *A. thaliana* expressing P30 suggests that the processed P30 was nonfunctional in supporting virus movement.

By an extended mutational analysis of the P30 MP, separate functional domains for RNA binding, cell wall/plasmodesmata targeting, increasing of plasmodesmatal SEL, and phosphorylation were found. Alignment of the amino acid sequences of tobamoviral MPs resulted in the identification of two conserved regions, denoted regions I (aa 56-96) and II (aa 125-165) (Saito et al., 1988). Mutations in region I affect the stability of P30 (Citovsky et al., 1992), binding of P30 to microtubules and targeting to plasmodesmata (Kahn et al. 1998). Region II is part of the domain required for enlargement of plasmodesmal SEL (aa 126-224; Waigmann et al., 1994) and overlaps with one of two stretches involved in RNA-binding (aa 112-185 and 185-268 (Citovsky et al., 1992)). The tobamoviral MPs contain many charged amino acids, most of which are localised in the C-terminal third. This region can be subdivided into three regions: a large region rich in basic amino acids (called region B) that is flanked by two smaller regions rich in acidic amino acids (regions A and C) (Saito et al., 1988). Mutational analysis has demonstrated that region C and most of region B are not important for cell-to-cell movement (Berna et al., 1991, Gafny et al., 1992). The functional importance of this part of the P30 protein remains unknown, but threonine-262 and serines 258 and 265 might be phosphorylated during infection (Citovsky et al., 1993, 1999). In addition, the MP sequence contains more serine residues between amino acids 61-114 and 212-231 that can be phosphorylated (Haley et al., 1995). Two other functional domains, one in the N-terminal region and the other comprising residues 185-224, have been implicated in the interaction with cellular pectin methylesterase (PME) (Citovsky, 1999, Chen et al., 2000). This protein may have a role in targeting of P30 to the cell wall or may function as a receptor molecule, regulating binding of P30-RNA complexes to plasmodesmata. The N-terminal PME-binding domain of P30 is essential for cell-to-cell movement, while deletion of the C-terminal phosphorylation domain results in virus slightly more infectious in *Nicotiana tabacum* than wild-type TMV (Gafny et al., 1992).

A model for P30-mediated cell-to-cell movement

In order to spread to neighbouring cells, TMV genomes must be transported from the sites of replication to plasmodesmata (Figure 2B). TMV RNA replication takes place at membraneous structures in the cytoplasm, which are referred to as viroplasms (Más and Beachy, 1999). These structures are derived from the endoplasmic reticulum and contain replicase but also MP as part of the replication complex (Heinlein et al., 1998, Más and Beachy, 1999). Possibly, binding between P30 and genomic RNA occurs directly after translation (Heinlein et al., 1998, Más and Beachy, 1999). Complexes consisting of MP, replicase and viral RNA are transported intracellularly along the ER and/or microtubules (Reichel et al., 1999; Más and Beachy, 1999, Boyko et al., 2000). It has been suggested that the viral RNA-MP complexes carries a plasmodesmata localisation signal for reaching their destiny. This putative signal might be located in domain of P30 that is required for increasing plasmodesmal SEL. Alternatively, unprocessed PME is transported to the cell wall via the ER (Gaffe et al., 1997) and may subsequently bind the MP complex and guide it to the plasmodesmata (Chen et al., 2000). Actin or specific docking proteins might be involved in docking of the viral RNA-MP complex to a plasmodesmal receptor (Kragler et al., 1998). This binding could initiate modification of plasmodesmal SEL, after which the viral RNA-MP complexes passes through the cell wall attached to the desmotubule (Más and Beachy, 1999). The actual translocation process across the plasmodesmata might be regulated by interactions between vRNP complex and components of a plasmodesmal trafficking apparatus, which is involved in cell-to-cell trafficking of host components. Individual components of this mechanism have not yet been isolated, but may include chaperonins, escort proteins, motor proteins and kinases that supply the necessary energy. Moreover, it appears likely that after translocation of viral RNA-MP complexes, there is a mechanism to control the activity of P30, that might involve phosphorylation of P30 (Citovsky, et al., 1993).

Other viruses moving as a nucleoprotein complex

The typical features postulated for the P30 MP of TMV, like (1) binding of single-stranded nucleic acids, (2) interaction with cellular components as the cytoskeleton and endoplasmic reticulum, (3) localisation to plasmodesmata, (4) ability to enlarge the plasmodesmal SEL, and (5) movement of the bound nucleic acids through plasmodesmata, have also been attributed to the MPs of several other groups of plant viruses, which are thought to move from cell-to-cell in a similar way. This group includes rod-shaped viruses as tobra- and furoviruses, isometric viruses like dianthoviruses and the class of geminate viruses (see Table 2).

Table 2: Overview of group II MPs

genus[a]	virus[b]	genome type	segments	particle morhology	CP[c]	MP
Tobamovirus	TMV	ssRNA	1	rod-shaped	-	30K[1]
Tobravirus	TRV	ssRNA	2	rod-shaped	-	1a[2]
Dianthovirus	RCNMV	ssRNA	2	isometric	-	35K[3]
Furovirus	*SbWMV*	*ssRNA*	*2*	*rod-shaped*	*?*	*p37*[4]
Begomovirus	SqLCV	ssDNA	2	geminate	-	NSP, MBT[5]
Curtovirus	*BCTV*	*ssDNA*	*2*	*geminate*	-	*R3*[6]
Mastrevirus	*MSV*	*ssDNA*	*1*	*geminate*	+	*V1*[7]

[a] Viruses that spread from cell-to-cell as a nucleoprotein complex through plasmodesmata with an increased SEL. These complexes are transported to plasmodesmata through interactions with the cytoskeleton and/or ER. Furoviruses are putatively classified in this group due to the homology of their MPs to other group II MPs and the mechanisms of intercellular spread employed by curto- and mastreviruses are thought to resemble that of begomoviruses.

[b] TMV, tobacco mosaic virus; TRV, tobacco rattle virus; RCNMV, red clover necrotic mosaic virus; SbWMV, soil-borne wheat mosaic virus; SqLCV, squash leaf curl virus; BCTV, beet curly top virus; MSV, maize streak virus

[c] Involvement of the CP in cell-to-cell movement. Key, +, genetic evidence provided for a CP requirement; -, genetic evidence that the CP is not involved; ?, not known

Key references: 1, Deom et al. (1987); 2, Hamilton et al. (1987); 3, Osman et al. (1991); 4, Shirako and Wilson (1994); 5, Sanderfoot and Lazarowitz (1995); 6, Hormuzdi and Bisaro (1993); 7, Boulton et al. (1993);

The SEL of plasmodesmata increases during tobravirus infection (Derrick et al., 1992). Mutational analysis of the tobacco rattle virus (TRV) genome identified the 29 kDa la protein as the viral MP (Hamilton and Baulcome, 1989). Unfortunately, further details on TRV cell-to-cell movement are scarce.

Like tobamoviruses, the isometric dianthoviruses, as RCNMV, do not require their CP for cell-to-cell spread (Xiong et al., 1993). By mutagenesis, a 35 kDa protein expressed by RCNMV RNA-2 has been identified as a MP (Osman et al., 1991). This protein can bind nucleic acids *in vitro* (Osman et al., 1992), an activity that was mapped to amino acids 181-225 (Osman et al., 1993). The results of co-injection studies with wild-type and mutant RCNMV MP suggested that the MP and viral MP-RNA complexes moved via plasmodesmata, similarly as proposed for TMV (Fujiwara et al., 1993).

The viruses belonging to the geminiviridae are subdivided on the basis of their genome organisation, insect vector and host range into three genera. Curto- and mastreviruses need both their MP and CP for cell-to-cell movement (Boulton et al., 1989; Briddon et al., 1989), while the begomoviruses do not require their CP for cell-to-cell movement (Ingham et al., 1995; Sudarshana et al., 1998). All geminiviruses replicate in the nucleus and therefore first need to cross an additional barrier: the nuclear envelope. Nuclear export of the genomes of the bipartite begomoviruses is regulated by the NSP protein

(formerly known as BR1; Lazarowitz and Beachy, 1999). NSP has a nuclear localisation and DNA-binding properties (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995), and binds to the newly formed genomes and exports them from the nucleus. In the cytoplasm, the ssDNA-NSP complexes interact with a second viral protein, MPB (also known as BL1 and BC1). This complex is targeted to the plasmodesmata by association with elements of the cortical ER (Ingham et al., 1995; Sanderfoot and Lazarowitz, 1995; Lazarowitz and Beachy, 1999). Movement of the ssDNA-NSP complex to neighbouring cells occurs via tubular structures. MPB is a structural component of these tubular structures, which are derived from the ER (Ward et al., 1997). The tubular structures could represent desmotubules that have been modified upon action of MPB or could be formed during cell wall formation (since geminiviruses like squash leaf curl virus (SqLCV) replicate in dividing phloem cells) (Lazarowitz and Beachy, 1999). Viral particles associated with these tubules have not been detected, emphasizing that the tubular structures are different from the ones found during comovirus infections. In the neighbouring cell, the MPB-ssDNA-NSP complex dissociates and the NSP subsequently transports the ssDNA into the nucleus, where a new round of genome amplification will start. The mechanisms used by the other two geminiviral genera, the curto- and mastreviruses, are not well understood. No sequence homology has been detected between the MPs of begomoviruses and the V1 protein of maize streak virus (MSV), which likely acts as its MP (Boulton et al., 1993; Dickinson et al., 1996). In addition, the beet curly top virus (BCTV; a curtovirus) R3 protein has some sequence homology to the MSV V1 protein and may act as a MP (Hormuzdi and Bisaro, 1993). The possibility that the MSV CP and V1 proteins act in a mechanism similar to the two begomoviral proteins needs to be investigated.

The triple gene block: not one but three MPs

Viruses of disparate genera use a set of three MPs coded by a so-called triple gene block (TGB) for cell-to-cell movement. TGB genes are found in the genomes of potex- (Beck et al., 1991), hordei- (Petty and Jackson, 1990), carla- (Zavriev et al., 1991), beny- (Bouzoubaa et al., 1986; originally classified as furoviruses (see also Torrance and Mayo, 1997)), pomo- (Scott et al., 1994) and pecluviruses (Herzog et al., 1994; Torrance and Mayo, 1997). The proteins encoded by the TGB vary somewhat in size among different viruses. The largest variation is found with TGBp1's, for which the molecular masses vary between 24 and 62 kDa. The other two TGB proteins are smaller, TGBp2's have molecular masses between 12 and 14 kDa and the molecular

weights of TGBp3's vary between 7-22 kDa. In addition to the three proteins of the TGB, some viruses also need their CP for cell-to-cell movement. Among the TGB-containing viruses (Table 3), the best studied viruses are the potexviruses potato virus X (PVX) and white clover mosaic virus (WClMV) and the hordeivirus barley stripe mosaic virus (BSMV).

Table 3: Overview of viruses that code for class III MPs

genus[a]	virus[b]	genome type	Segments	particle morhology	CP[c]	MPs
Potexvirus	PVX	ssRNA	1	filamentous	+	TGBp1, TGBp2, TGBp3[1]
Carlavirus	PVM	ssRNA	1	filamentous	+	TGBp1, TGBp2, TGBp3[2]
Benyvirus	BNYVV	ssRNA	5	rod-shaped	-	P42, P13, P15[3]
Hordeivirus	BSMV	ssRNA	3	rod-shaped	-	βb, βc, βd[4]
Pecluvirus	PCV	ssRNA	5	rod-shaped	-	TGBp1, TGBp2, TGBp3[5]
Pomovirus	*PMTV*	*ssRNA*	*3*	*rod-shaped*	-	*P51, P13, P21*[6]
Allexivirus	*ShVX*	*ssRNA*	*1*	*rod-shaped*	*?*	*ORF2, ORF3, ORF4*[7]
Foveavirus	*ASPV*	*ssRNA*	*1*	*filamentous*	*?*	*TGBp1, TGBp2, TGBp3*[8]

[a] Viruses that code for a triple gene block, a set of three MPs. For groups shown in *italics* only sequence information is available

[b] PVX, potato virus X; PVM, potato virus M; BSMV, barley stripe mosaic virus; PCV, peanut clump virus; PMTV, potato mop-top virus; ShVX, shallot virus X; BNVYY, beet necrotic yellow vein virus; ASPV, apple stem pitting virus

[c] Involvement of the CP in cell-to-cell movement. Key, +, genetic evidence provided for a CP requirement; -, genetic evidence that the CP is not involved; ?, unknown

Key references: 1, Beck et al. (1991); 2, Zavriev et al. (1991); 3, Bouzoubaa et al. (1986) 4, Petty and Jackson (1990); 5, Herzog et al. (1994); 6, Scott et al. (1994); 7, Kanyuka et al. (1992); 8, Jelkmann (1994)

Involvement of the TGB proteins in cell-to-cell movement

The three TGB proteins are expressed from two subgenomic RNAs; the TGBp1 protein is expressed from a monocistronic mRNA molecule, whereas the other two proteins are translated from a single bicistronic mRNA (Morozov et al., 1991b; Gilmer et al., 1992; Zhou and Jackson, 1996; Verchot et al., 1998). The TGBp3 gene is translated by leaky ribosome scanning through the TGBp2 mRNA (Zhou and Jackson, 1996; Verchot et al., 1998).

All three TGB proteins are needed for cell-to-cell movement, as has been shown for different viruses by deletion analysis (Petty and Jackson, 1990; Beck et al., 1991, Gilmer et al., 1992; Herzog et al., 1998). Though all three TGB proteins are involved in virus cell-to-cell movement, they differ in cellular location. Potexviral TGBp1 has been immunolocalised to virus-induced inclusions in the cytoplasm but was not found in plasmodesmata (Davies et al., 1993; Rouleau et al., 1994; Chang et al., 1997; Kalinina et al., 1998). In contrast to potexviral TGBp1, TGBp1's of hordei- and pecluviruses were detected in cell wall fractions by Western blotting (Niesbach-Klösgen et al., 1990;

Donald et al., 1993; Erhardt et al., 1999) and more recently, the TGBp1 protein (P51) of the pecluvirus peanut clump virus (PCV) was found to occur in plasmodesmata by immunolabeling (Erhardt et al., 1999). The plasmodesmata of PCV-infected cells did not show any obvious morphological alterations in comparison with those of non-infected cells. In contrast to these results, Santa Cruz et al. (1998) reported the appearance of a fibrillar material in plasmodesmata of cells infected with PVX. In most cases these plasmodesmata did not contain a recognisable desmotubule and had a distorted appearance. Immunogold labeling using specific antisera showed that PVX CP was associated with these fibrillar structures (Santa Cruz et al., 1998). Recently it has been reported that fusion products of the benyviral beet necrotic yellow vein virus (BNYVV) TGBp1 (P42) protein and GFP localised to punctate bodies in the cell wall which presumably are plasmodesmata (Erhardt et al., 2000). Accumulation of P42 in these bodies was dependent on the presence of both P13 (TGBp2) and P15 (TGBp3). Point mutations in P42 that inhibited cell-to-cell spread of BNYVV also inhibited the formation of the punctate bodies.

Amino acid sequence analysis revealed that TGBp1's contain a sequence motif corresponding to a NTPase domain found in helicases of viruses belonging to the sindbis virus-like supergroup (Gorbalenya and Koonin, 1989). Indeed, TGBp1 of PVX, BSMV and several other viruses have been shown to bind ATP and possess ATPase activity *in vitro* (Kalinina et al., 1996; Donald et al., 1997). This quality might be important *in vivo*, since deletions in the NTPase domain blocked cell-to-cell movement (Donald et al., 1997; Bleykasten et al., 1996). Besides TGBp1's of potexviruses, also the beny- and hordeiviruses homologues have been shown to bind nucleic acids *in vitro*, but the RNA-binding is rather variable in strength for different viruses (Rouleau et al., 1994; Bleykasten et al., 1996; Kalinina et al., 1996; Donald et al., 1997; Wung et al., 1999). By mutational analysis it was then shown that the N-terminal part of the PVX TGBp1 is responsible for both the ATPase activity and the RNA-binding (Morozov et al., 1999). Wung et al. (1999) mapped the RNA-binding domain of the potexvirus bamboo mosaic virus (BaMV) TGBp1 between amino acids 3 and 24. In particular, three arginine residues present in this region are essential for RNA-binding. At the other hand, BSMV βb (TGBp1) appeared to contain multiple RNA-binding domains, distributed over the N- and C-terminal regions of the protein (Donald et al., 1997). The RNA-binding domain of the BNYVV P42 protein (TGBp1) has again been localised to the N-terminus of the protein, between amino acids 2-49 (Bleykasten et al., 1996). The fact that a mutant BNYVV virus that carries a deletion in the N-terminus of TGBp1 is not able to infect plants but is able to replicate in protoplasts (Bleykasten et al., 1996), supports the

idea that the nucleic acid-binding activity of TGBp1 detected *in vitro* is of functional significance for virus movement *in vivo*.

Micro-injection experiments have demonstrated that cell-to-cell movement of PVX is accompanied by an increase in plasmodesmal SEL of at least 10 kDa and that the TGBp1 protein is responsible for this increase (Angell et al., 1996; Lough et al., 1998). Micro-injected WClMV TGBp1 can spread to neighbouring cells by itself, but movement of the viral RNA is only accomplished in the presence of TGBp1, TGBp2, TGBp3 and CP (Lough et al., 1998).

Both TGBp2 and TGBp3 possess two hydrophobic domains and have affinity for membranes *in vitro* (Morozov et al., 1990) and *in vivo* (Niesbach-Klösgen et al., 1990; Morozov et al., 1991a, 1991; Donald et al., 1993). PVX TGBp3 has been immunolocalised to the plasma membrane and the cell wall but has not been found in plasmodesmata (Hefferon et al., 1997). GFP-tagged TGBp2 encoded by the hordeivirus poa semilatent virus (PSLV) was associated with elements of the endomembrane system and GFP-TGBp3 was found in peripheral membrane bodies (Solovyev et al., 2000). Site-directed mutagenesis showed that the transmembrane segments of TGBp2 and the central hydrophilic region of TGBp3 are responsible for the intracellular targeting of TGBp2 and TGBp3, respectively. The intracellular location of TGBp2 dramatically changed in cells co-expressing TGBp3: TGBp2 now appeared in peripheral bodies also found in cells transiently expressing TGBp3 alone (Solovyev et al., 2000). Since none of the mutations introduced in TGBp2 had any effect on the TGBp3-driven accumulation of TGBp2 in peripheral bodies, specific interactions between TGBp2 and TGBp3 are probably not involved in this process. Possibly, TGBp3 activates a cellular pathway that is responsible for the transport of TGBp2 to the peripheral bodies.

Potex- and carlaviruses, but not hordei- and pecluviruses, moreover require their CPs for cell-to-cell movement (Chapman et al., 1992; Santa Cruz et al., 1998; Petty and Jackson, 1990; Herzog et al., 1998). The requirement for a CP might be correlated with the occurrence of fibrillar structures in plasmodesmata in infected cells, as shown for PVX (Oparka et al., 1996; Santa Cruz et al., 1998). The CPs of several other potexviruses were also found in plasmodesmata of virus-infected cells (Rouleau et al., 1995). The CP does not contribute to the modification of the plasmodesmal SEL, and its precise function in virus cell-to-cell movement is not yet clear (Oparka et al., 1996; Santa Cruz et al., 1998). At present, there are two opposing views about the role of the CP in potexviral cell-to-cell movement (Figure 2C). Based on the results of micro-injection experiments Lough et al. (1998) suggested that a non-virion complex, containing viral RNA, TGBp1 and CP, is involved in cell-to-cell movement of WClMV. On the other

hand, Santa Cruz et al. (1998) claimed, using antisera specifically recognising PVX particles, that virus particles are present within plasmodesmata of infected cells, suggesting that the flexous PVX particles (which are 515 nm long and have a diameter of 13 nm) can be transported through modified plasmodesmata. The latter results are in agreement with the early report of Allison and Shalla (1974), who described fibrillar material which might represent viral particles in plasmodesmata of PVX-infected cells .

TGBp1 proteins of hordei-, beny-, pomo- and pecluviruses are larger than those of potex- and carlaviruses with an N-terminal extension of 20-40 kDa (Bleykasten et al., 1996; Wong et al., 1998). Remarkably, this division coincides with the differences in requirement for CP for cell-to-cell movement. The N-terminal extension of hordeiviral TGBp1's contains clusters of positively charged redidues that have a role in RNA-binding (Solovyev et al., 1996).
The TGBp3 proteins can likewise be divided in two groups (Solovyev et al., 1996) in which peclu-, pomo-, and hordeiviral TGBp3 proteins (group I) are significantly larger than those of potex- and carlaviruses (group II): their sizes are in the range of 14-22 kDa and 6-8 kDa, respectively. TGBp3 proteins of groups I and II do not share significant sequence homology and differ in the number of putative transmembrane domains, i.e. group I proteins contain two transmembrane domains and proteins classified in group II only one (Morozov et al., 1991a). The central, hydrophilic region conserved in group I proteins is responsible for the delivery of PSLV TGBp3 to peripheral membrane compartments (Solovyev et al., 2000).
The TGBp2 proteins are not subdivided in such a way. Comparison of TGBp2 proteins of potexviruses with the corresponding proteins in hordei-, peclu- and carlaviruses reveals a similar organisation, with two blocks of hydrophobic residues separated by a strongly conserved hydrophilic region (Solovyev et al., 1996). The C-terminal parts of these proteins are relatively rich in cysteine residues that may be involved in transport and insertion of the proteins into the plasma membrane (Solovyev et al., 1996).

A model of TGB-mediated cell-to-cell movement

So, in what respect does the mechanism of cell-to-cell movement utilised by TGB-containing viruses differ from the earlier described mechanisms used by como- and tobamoviruses respectively? Although the details of the mechanism that TGB-containing viruses use for their cell-to-cell movement are still unknown, different properties of the TGB proteins suggest that these viruses use a mechanism with similarities to that of tobamoviruses. In their occurrence in plasmodesmata, their ability to increase the SEL of plasmodesmata and to bind nucleic acids, TGBp1's have several characteristics in

common with the TMV P30 MP. It is possible that the functions encoded by the single MP of TMV are distributed over three proteins in viruses using TGB proteins. TGBp1 then would code for core functions like increasing the plasmodesmal SEL and binding to the viral RNA), while TGBp2 and TGBp3 would have specific but yet undefined roles in transporting the complexes of viral RNA to the plasmodesmata. Potex- and carlaviruses cannot spread from cell-to-cell without CP. Since the PVX TGBp1 protein has only weak RNA-binding properties, the CP may support the role of TGBp1 as a specific RNA-binding protein. Lough et al. (1998) presented a model for cell-to-cell movement of WClMV in which a RNP complex of viral RNA, TGBp1 and CP is transported via the cytoskeleton to plasmodesmata (Figure 2C). TGBp3 may target TGBp2 to the cell periphery, where TGBp2 may direct TGBp1 and/or RNP complexes to membrane compartments close to the cell wall or plasmodesmata to enable docking of the RNP complex to a putative plasmodesmal receptor (Lough et al., 1998, Solovyev et al., 2000). TGBp1 increases the plasmodesmal SEL, enabling transport of the RNP complex to an adjacent cell.

On the other hand, Santa Cruz et al. (1998) take the view that potexviruses are translocated across plasmodesmata as virus particles. They suggested that in PVX-infected cells the desmotubule is removed from the plasmodesmata, probably by action of TGBp1, resulting in an opening (> 20 nm) large enough to allow passage of the flexuous PVX particles (Figure 2C). In plants infected with PVX or other TGB-containing viruses tubular structures have never been found, neither has it been shown that TGBp's have tubule-forming capacities in protoplasts. This points to essential differences with the mechanism for cell-to-cell movement used by comoviruses.

In which conditions each of the two mechanisms described above is used, is at the moment unclear. However, Santa Cruz et al. (1998) found indications that these mechanisms may be tissue-specific. They detected CP in plasmodesmata between all cell types except in those between companion cells and sieve elements and those between phloem parenchyma and companion cells, suggesting that PVX may spread between these cell types as viral nucleoprotein complexes and not as viral particles.

Viruses using ambiguous movement mechanisms

Movement of the alfamo-, ilar-, bromo-, olea- and cucumoviruses, all belonging to the family of Bromoviridae, seems rather ambiguous, as these viruses seem able to spread from cell-to-cell via two different mechanisms. The genome of the Bromoviridae consists of three positive sense, single-stranded RNA molecules which are encapsidated

in separate particles. Bromo- and oleaviruses have isometric particles with a diameter of 25-28 nm and alfamo- and ilarviruses have bacilliform particles with a length of 30-56 nm and a width of 18 nm. Cucumoviral particles are isometric with a diameter of 30 nm. The viral MPs are encoded by RNA3 and show characteristics of the MPs of both como- and tobamoviruses. In addition to the MP, most Bromoviridae need their CP for cell-to-cell movement (Suzuki et al., 1991, Van der Kuyl et al., 1991, Rao and Grantham, 1995). We will describe the cell-to-cell movement of AMV as the most typical example of a virus of this group in more detail and then compare the data available for the other groups with those of AMV.

Involvement of AMV-P3 in cell-to-cell movement

A role of the protein P3, encoded by RNA3, in cell-to-cell movement was first suggested after the detection of P3 in the cell wall of AMV-infected tobacco plants using P3-antisera (Godefroy-Colburn et al., 1986; Stussi-Garaud et al., 1987). Subsequently, deletion analysis of RNA3 genetically identified P3 as the AMV MP (Van der Kuyl et al., 1991). P3 has several characteristics in common with the TMV MP. Like P30, P3 is able to increase the SEL of plasmodesmata. Poiron et al. (1993) established by micro-injection of dextran molecules that the SEL of plasmodesmata in transgenic plants expressing P3 is larger then in non-transgenic plants. The increase in size of plasmodesmata of P3-expressing plants allowed spread of a 4.4 kDa dextran, which is much less than for plants expressing TMV-P30, in which dextrans with a molecular weight of 9.4 kDa diffused from cell-to-cell. Furthermore, UV-crosslinking assays and electrophoretic retardation experiments showed that P3 also has the ability to bind to single-stranded nucleic acids *in vitro* (Schoumacher et al., 1992, 1994). Recent data data obtained by Huang and Zhang (1999) show that a fusion product of P3 and GFP colocalises with the ER in tobacco and onion cells. Moreover, subcellular fractionation and immunoblotting analysis suggested that P3 acts like an integral membrane protein. These data indicate that P3 might use the ER for intercellular movement (Huang and Zhang, 1999).

In addition, tubular structures were found in cowpea protoplasts inoculated with AMV (Kasteel et al., 1997). Both bacilliform and isometric AMV particles were detected inside the tubular structures in infected protoplasts. Similar tubular structures were found in protoplasts transiently expressing P3-GFP (Zheng et al., 1997). These results indicate that AMV P3 is capable of forming tubules enclosing virus particles. Furthermore, AMV also requires CP for cell-to-cell movement (Van der Kuyl et al., 1991). By immunogold labeling using specific antisera both P3 and CP were localised to plasmodesmata (Van der Wel et al., 1998). The plasmodesmata of infected cells appeared modified in that

the desmotubule was absent while the mean diameter of these plasmodesmata (36 nm) was almost twice the mean diameter of plasmodesmata in uninfected cells (20 nm). Both the plasmodesmal localisation of CP and P3 and the increase in plasmodesmal size were only observed during a short period early in the infection process (Van der Wel et al., 1998). These results correspond with the finding that P3 is only transiently expressed during early phases of virus infection (Berna et al., 1986; Stussi-Garaud et al., 1987). No tubular structures containing virus particles were found associated with these modified plasmodesmata, so if there are tubules, these must be very short and only span the plasmodesmal channel (Van der Wel et al., 1998).

By mutational analysis of P3 different regions have been identified that are important for functioning of the MP. Using western blotting of proteins from AMV-infected and P3-transgenic plants it was shown that the region between amino acids 21-35 of P3, which most likely will be folded into an α-helical structure, is involved in targeting of P3 to the cell wall (Erny et al., 1992; Berna, 1995). Gel-retardation and cross-linking experiments indicated a RNA-binding domain located between residues 36 and 77 (Schoumacher et al., 1994). By deletion analysis, Poirson et al. (1993) established that the N-terminal 77 amino acids of P3 have no role in increasing the plasmodesmal SEL. Preliminary results suggest that viruses coding for MPs lacking amino acids 21-34 or 36-81 are able to spread in tobacco, suggesting that the regions of P3 thought to have functions in cell wall targeting and nucleic-acid binding are dispensable for cell-to-cell movement *in planta* (Giovane et al., 1994). Large parts of P3 are presumably involved in tubule formation, since deletions in the regions 1-77, 84-142 and 226-300 blocked tubule formation (Zheng et al., 1997). Amino acids 87-100 encompass a 30K-superfamily domain, a stretch of 32 amino acids that is conserved in the MPs of 20 plant virus genera including como- and tobamoviruses (Melcher, 2000).

A model for AMV cell-to-cell movement

P3, the AMV MP, has characteristics of the MPs of CPMV as well as that of TMV and until now, it is not clear whether AMV uses a TMV-like mechanism, a CPMV-like mechanism, or both mechanisms for cell-to-cell movement (Figure 2D). A TMV-like mechanism would involve binding of AMV RNA to P3 molecules in the cytoplasm and guidance of these viral RNA-P3 complexes via the endoplasmic reticulum to plasmodesmata (Huang and Zhang, 1999). There, P3 modifies the plasmodesmal SEL and directs translocation of these complexes to neighbouring cells. A role for the CP in this mechanism is unclear. The second mechanism might involve a CPMV-like transport of AMV particles through tubular structures (Kasteel et al., 1997; Van der Wel et al., 1998). Since there are no clear tubular structures visible in the modified plasmodesmata

of infected plants, these tubules probably are relatively short, spanning only the plasmodesmal channel (Van der Wel et al., 1998). Each of these two mechanism might be used in specific host plants or for cell-to-cell movement in certain tissues, although such preferences for either mechanism are not known.

Table 4: Viruses coding for class IV MPs

genus[a]	virus[b]	genome type	segments	Particle morphology	CP[c]	MP
Alfamovirus	AMV	ssRNA	3	*Bacilliform*	+	P3[1]
Ilarvirus	TSV	ssRNA	3	Bacilliform	+?	P3[2]
Bromovirus	BMV	ssRNA	3	Isometric	±	3a[3]
Cucumovirus	*CMV*	*ssRNA*	*3*	*Isometric*	+	*P3*[4]
Oleavirus	OLV-2	ssRNA	3	Isometric	+?	36K[5]
Idaeovirus	*RBDV*	*ssRNA*	*2*	*Isometric*	*?*	*p39*[6]

[a] Viruses that use ambiguous movement mechanisms with characteristics of the mechanisms developed by group I and II MPs. The evidence for idaeoviruses (shown in *italics*) is based on sequence homology studies.

[b] AMV, alfalfa mosaic virus; TSV, tobacco streak virus; BMV, brome mosaic virus; CMV, cucumber mosaic virus; OLV-2, olive latent virus-2; RBDV, raspberry bushy dwarf virus

[c] Involvement of the CP in cell-to-cell movement. Key, +, genetic evidence provided for a CP requirement; ±, some members of the genus require their CP, others not; -, genetic evidence that the CP is not involved; +?, no genetic evidence available for a CP-requirement, but probably needs CP; ?, unknown

Key references: 1, Van der Kuyl et al. (1991); 2, Cornelissen et al. (1984); 3, Schmitz and Rao (1996); 4, Suzuki et al. (1991); 5, Grieco et al. (1995); 6, Natsukai et al. (1991)

Characteristics of the MPs of other Bromoviridae

AMV P3 shows amino acid sequence homology to the MPs of bromo-, ilar- and oleaviruses, and to a lesser extent to the MP of cucumoviruses (Koonin et al., 1991). Whether all viruses of these groups have developed similar mechanisms for cell-to-cell movement is still unclear, although most of the MPs of these viruses have characteristics similar to P3.

Details about the cell-to-cell movement of ilarviruses are lacking, but presumably the ilarviruses spread from cell-to-cell just like AMV, since the MP of the ilarvirus prunus necrotic ringspot virus (PNRSV) and AMV have 52% similar amino acids (see Sánchez-Navarro and Pallás, 1997). Furthermore, chimeric viruses in which the AMV CP or MP were replaced by the corresponding protein of PNRVS are able to spread in inoculated tobacco plants, albeit with a reduced efficiency (Sánchez-Navarro et al, 1997).

For bromoviral cell-to-cell movement similar observations have been made as described above for AMV. The MP was initially identified by mutagenesis of the BMV genome (Schmitz and Rao, 1996). It can bind to single-stranded nucleic acids *in vitro*, similar to

AMV P3 (Fujita et al., 1998; Jansen et al., 1998). Deletion mutagenesis located a nucleic-acid binding domain to residues 189-242 (Fujita et al., 1998). The functionality of this domain *in vivo* is not yet clear. Immunogold labeling using anti-MP serum located the BMV MP in cytoplasmic inclusions which might be derived from the endoplasmic reticulum and in plasmodesmata (Hosokawa et al., 1992; Fujita et al., 1998). Just like AMV, BMV is able to induce the formation of tubular structures containing virus particles in protoplasts (Kasteel et al., 1997). And, as for AMV, no tubular structures have been detected in BMV-infected tissue (Fujita et al., 1998). Cowpea chlorotic mottle virus (CCMV) may be an exceptional bromovirus in that, in contrast to BMV, it can spread from cell-to-cell in the absence of functional CP (Rao and Grantham, 1995; Schmitz and Rao, 1996, Schneider et al., 1997)

While *in planta* the AMV MP, and likely also the BMV MP, might achieve the formation of short tubular structures, in plants infected with the oleavirus olive latent virus-2 (OLV-2), long tubular structures containing virus-like particles have been detected (Castellano et al., 1987). In protoplasts, the OLV-2 MP is able to form tubular structures in the absence of other viral proteins (Grieco et al., 1999). Until now RNA-binding by the OLV-2 MP has not been studied, but, as the OLV-2 MP has amino acid sequence homolgy to the bromo- and cucumovirus MPs (Grieco et al., 1995), it likely has this property.

The cucumber mosaic virus (CMV: type member of the cucumoviruses) MP was shown by deletion analysis to be encoded by the 3a protein (Suzuki et al., 1991). This protein binds nucleic acids *in vitro* (Li et al., 1996) and is localised to plasmodesmata (Vaquero et al., 1994). Micro-injection experiments revealed that 3a increased the SEL of plasmodesmata to 2.3 nm, sufficient to allow transport of viral RNA-3a complexes (Vaquero et al. 1993). The 3a protein can form tubular structures in protoplasts reminiscent to the structures formed by AMV and OLV-2 (Canto and Palukaitis, 1999). However, the finding of two CMV mutants that do not form tubules in protoplasts but are able to spread *in planta*, suggests that the tubules do not have an essential role in cell-cell transport (Canto and Palukaitis, 1999). CMV requires its CP for cell-to-cell movement (Suzuki et al., 1991, Canto et al., 1997), but does not spread from cell-to-cell as virus particles since CMV mutants unable to encapsidate the viral RNA were able to spread intercellularly (Kaplan and Palukaitis, 1998, Schmitz and Rao, 1998). Furthermore, virus particles could not be detected in plasmodesmata by immunocytochemistry (Ding et al., 1995; Blackman et al., 1998), although some older reports claimed the occurrence of virus-like particles in modified plasmodesmata visualised by electron microscopy (Lawson and Hearon, 1970, Martelli and Russo, 1985). The current idea is that CMV spreads intercellularly using a mechanism corresponding to that of TMV except that the ribonucleoprotein complex that is

translocated across the cell wall consists of viral RNA, MP and CP (Blackman et al., 1998). Since CP and tubular structures have not been detected in plasmodesmata, and a defect in tubule-forming capacities in protoplasts does not affect spread *in planta*, CMV probably does not spread from cell-to-cell via a tubule-guided mechanism.

Other types of viral movement proteins

Besides the four types of MPs discussed before, it is possible to distinguish at least 6 other types of MPs, which we shall briefly describe in this section (Table 5). Potyviruses (1), carmo- and necroviruses (2), closteroviruses (3) and poleroviruses (4) all encode MPs which have unique characteristics that requires classification in separate groups. Potyviruses do not possess a classical MP only involved in cell-to-cell spread, but need three proteins for their cell-to-cell movement. The MPs of carmo- and necroviruses are very small compared to other MPs and closteroviruses require five proteins for cell-to-cell movement. Intercellular spread of the phloem-limited poleroviruses is regulated by a protein that is not related to other viral MPs.

1. Potyviruses (class V)

The potyviruses make up a large group of filamentous viruses that do not encode a specific MP with a primary function in cell-to-cell movement. Instead, three proteins have been implicated in cell-to-cell movement which also have other well-defined functions. The CI protein (CI stands for cylindrical inclusion) is involved in viral replication (Klein et al., 1994). HC-Pro is a proteinase (Pro) involved in polyprotein cleavage that also has a role as helper-component (HC) in aphid transmission and a function in long-distance movement (reviewed in Maia et al., 1996). As many other viruses, potyviruses also require their CP for cell-to-cell movement.

In cells infected with potyviruses, large pinhweel-like structures can be found on both sides of the cell wall that seem to be formed at modified plasmodesmata (Roberts et al., 1998). These structures consist of a cone of triangular protein plates attached to a central tubule. The opening of the tubule might align with plasmodesmal openings (Rodríguez-Cerezo et al., 1997, Roberts et al., 1998). Using immunogold-labeling, Rodríguez-Cerezo et al (1997) showed that the CI protein was specifically localised to these structures. Furthermore, also CP and viral RNA were detected at these plasmodesmata-associated structures. Genetic evidence for a role of CI in viral movement was acquired by Carrington et al. (1998), who showed that two TEV mutants

with substitutions in the N-terminal region of the CI protein were unable to spread from cell-to-cell in plants but were able to replicate similarly as wild-type virus in protoplasts. HC-Pro also has been implicated in cell-to-cell movement, where it may function in the enlargement of plasmodesmal SEL. Micro-injection studies showed that bean common mosaic necrosis virus (BCMNV) and lettuce mosaic virus (LMV) HC-Pro can increase the SEL to 37 kDa and are able to traffic themselves and viral RNA from cell-to-cell in the absence of CI and CP (Rojas et al., 1997). In addition, HC-Pro facilitates the spread of co-injected CP, suggesting that these HC-Pro and CP may interact during cell-to-cell movement (Rojas et al., 1997). HC-Pro binds non-specifically to single-stranded nucleic acids (Maia and Bernardi, 1996), although a role for this activity in cell-to-cell movement remains unclear.

Dolja et al. (1994, 1995) obtained genetic evidence that the TEV CP is involved in cell-to-cell movement, as the infection of several mutant viruses with alterations in their CP coding region was restricted to single cells. BCMNV and LMV CPs also have the ability to increase the plasmodesmal SEL, although to a lesser extent than HC-Pro (Rojas et al., 1997). The form of the CP that is involved in cell-to-cell movement is not clear, although Dolja et al. (1994) obtained indications that cell-to-cell movement of TEV requires assembly-competent CP, suggesting that intercellular transport involves virion formation. In infected cells, CP was detectable, often in linear arrays, in the channel passing through the center of the pinwheel-like structure and in plasmodesmata (Rodríguez-Cerezo et al., 1997). The close proximity of CP and CI suggest that these two may interact specifically with each other.

How do all these data fit into a model for potyviral cell-to-cell movement? Probably, after replication viral genomes associate with CP either as a virion or as a ribonucleoprotein complex. These associate with the central channels of plasmodesmata-localised pinwheel structures composed of CI and are guided through these structures to neighbouring cells (Carrington et al., 1998). CI might function in the precise positioning of virus particles or ribonucleoprotein complexes for translocation across the cell wall. The CP and/or HC-Pro might increase the SEL of plasmodesmata to allow transfer of virions or ribonucleoprotein complexes.

2. Carmo- and necrovirus-like movement (class VI)

Carmo- and necroviruses have isometric particles with a diameter between 26 and 34 nm. Their genome consists of a single, positive-sense RNA molecule that encodes a set of two or three MPs, located centrally on the genome (Hacker er al., 1992; Molnár et al., 1997; Pantaleo et al., 1999). Because of the small sizes of these proteins (around 7-9 kDa) and the lack of amino acid sequence homology with other known MPs (Weng and

Xiong, 1997) they are classified in a separate group. Mutational analysis of the carmovirus turnip crinkle virus (TCV) p8 and p9 ORFs has shown that both proteins are needed for cell-to-cell movement (Li et al., 1998).

Table 5: Other types of viral movement proteins

genus	class[a]	virus[b]	genome type	segments	particle morphology	CP[c]	MP
Potyvirus	5	PVY	ssRNA	1	filamentous	+	CI, HC-Pro[1]
Bymovirus	*5*	*BYMV*	*ssRNA*	*2*	*filamentous*	*+?*	*CI, HC-Pro*[2]
Ipomovirus	*5*	*SPMMV*	*ssRNA*	*1*	*filamentous*	*+?*	*CI, HC-Pro*[3]
Macluravirus	*5*	*MacMV*	*ssRNA*	*1*	*filamentous*	*+?*	*CI, HC-Pro*[4]
Rymovirus	*5*	*RGMV*	*ssRNA*	*1*	*filamentous*	*+?*	*CI, HC-Pro*[5]
Tritimovirus	*5*	*WSMV*	*ssRNA*	*1*	*filamentous*	*+?*	*CI-HC-Pro*[6]
Necrovirus	6	TNV-D	ssRNA	1	isometric	+	p7ı, p7A, p7B[7]
Carmovirus	6	TCV	ssRNA	1	isometric	+/-	p8, p9[8]
Panicovirus	*6*	*PMV*	*ssRNA*	*1*	*isometric*	*?*	*p8, p8-FS, p6.6*[9]
Avenavirus	*6*	*OCSV*	*ssRNA*	*1*	*isometric*	*?*	*?*[10]
Machlomovirus	*6*	*MCMV*	*ssRNA*	*1*	*isometric*	*?*	*p8?*[11]
Closterovirus	7	BYV	ssRNA	1	filamentous	+	p65, p64, p6[12]
Crinivirus	*7*	*LIYV*	*ssRNA*	*2*	*filamentous*	*?*	*p65, p64, p6*[13]
Polerovirus	8	PLRV	ssRNA	1	isometric	?	p17[14]
Luteovirus	*8*	*BYDV*	*ssRNA*	*1*	*isometric*	*?*	*p17*[15]

[a] For details see main text. The virus groups presented in *italic* are putatively grouped in the classes 5, 6 and 7, based on amino acid seqence homology.

[b] PVY, potato virus Y; BYMV, beet yellow mosaic virus; SPMMV: sweetpotato mild mottle virus; MacMV, maclura mosaic virus; RGMV, ryegrass mosaic virus; WSMV, wheat streak mosaic virus; TNV-A, tobacco necrosis virus A; TCV, turnip crinkle virus; PMV, panicum mosaic virus; OCSV, oat chlorotic stunt virus; MCMV, maize chlorotic mottle virus; BYV, beet yellows virus; LIYV, lettuce infectious yellows virus; PLRV, potato leafroll virus

[c] Involvement of the CP in cell-to-cell movement. Key: +, genetic evidence provided for a CP requirement; -, genetic evidence that the CP is not involved; +?, no genetic evidence available for a CP-requirement, but probably needs CP; ?, unknown

Key references: 1, Carrington et al. (1998); 2, Guyatt et al. (1996); 3, Colinet et al. (1998); 4, Badge et al. (1997); 5, Salm et al. (1996) partial sequence; 6, Stenger et al. (1998); 7, Molnár et al. (1997); 8, Li et al. (1998); 9, Turina et al. (1998); 10, Boonham et al. (1995); 11, Nutter et al. (1989); 12, Alzhanova et al. (2000); 13, Klaassen et al. (1995); 14, Tacke et al. (1993); 15, Chay et al. (1996).

The TCV p8 protein, and the corresponding p7 protein of carnation mottle virus (CarMV), have RNA-binding properties (Wobbe et al., 1999; Marcos et al., 1999). The RNA-binding domain of the CarMV p7 MP was mapped to a centrally localised region of 16 mostly basic amino acids (Marcos et al., 1999). The second MP, p9, is rather hydrophobic and may be targeted to plasmodesmata (Marcos et al., 1999). In some hosts, also the TCV CP is required for cell-to-cell spread (Laakso and Heaton, 1993; Marcos et al., 1999).

Necroviruses as tobacco necrosis virus (TNV) strain A code for two proteins with a similar size and limited sequence similarity as the TCV MPs (Meulewaeter et al., 1990). Molnár et al. (1997) provided genetic evidence for a role of these two proteins in cell-to-cell movement of TNV strain D^H. In addition, they found that TNV strain D^H also needs its CP and the product of a third small ORF, $p7_1$, for intercellular movement.

3. Closteroviruses (Class VII)

Beet yellows virus (BYV) is the type member of the genus closterovirus, a group of filamentous viruses with genome consisting of a single, positive-sense RNA molecule. Using a BYV-variant expressing GFP as a reporter, Alzhanova et al. (2000) showed that BYV requires 5 proteins for its cell-to-cell spread, all of which are not necessary for RNA replication. Two are structural proteins, the others a 6 kDa hydrophobic protein, a 64-kDa protein (p64) that exhibits no significant similarity to any protein in the database, and a homolog of HSP70 molecular chaperones (p65) (Agranovsky et al., 1991; Koonin et al., 1991). This protein has microtubule-binding characteristics, which are enhanced by hydrolysis of ATP (Karasev et al., 1992). A domain involved in the ATPase activity is localised in the N-terminal part (Agranovsky et al, 1997). P65 has been detected in close proximity of plasmodesmata and inside plasmodesmal channels, and may be involved in the intercellular movement of BVY particles (Medina et al., 1999).

4. Poleroviruses (class VIII)

The poleroviruses are a group of phloem-limited viruses, previously classified as luteoviruses, that have isometric particles with a diameter of 23-30 nm. Although at present there is no genetic evidence that p17, encoded by ORF4, acts as a MP, its biochemical characteristics justifies such a classification. Furthermore, there is genetic evidence showing that the barley yellow dwarf virus (BYDV, classified in the genus luteovirus) p17 protein is required for cell-to-cell movement (Clay et al., 1996). Polerovirus p17 has RNA-binding properties (Tacke et al., 1991) and is phosphorylated in plants (Tacke et al., 1993). Residues located in an amphipathic α-helix in the N-terminal part of p17 are involved in oligomerisation of this protein (Tacke et al. 1993). RNA-binding properties are located in the C-terminal area (Tacke et al., 1991). P17 has been localised in plasmodesmata in infected tissue and in transgenic plants expressing p17 (Schmitz et al., 1997). P17 is phosphorylated by a membrane-associated protein kinase that has characteristics similar to protein kinase C (Sokolova et al., 1997).

Unclassified viral MPs

In addition to the MPs of virus genera described above, MPs have also been identified for several other groups of plant viruses but, due to lack of sufficient data, have not been classified in one of the earlier described classes (Table 6). Among these are the tombus- and aureusviruses, which encode only a single MP, with a molecular weight between 22 and 27 Da (Scholthof et al., 1995). Since the tombusvirus tomato bushy stunt virus (TBSV) does not require its CP for cell-to-cell movement (Scholthof et al., 1993), it is presumed that TBSV spreads as a ribonucleoprotein particle, similar to TMV. However, until more details of tombusvirus spread becomes available they are not classified in group I .

Until now, no MP has been identified for alphacrypto-, betacrypto-, cytorhabdo-, marafi-, ophio-, oryza-, ourmia- and phytoreoviruses.

Table 6: Unclassified viral MPs

genus	virus[a]	genome type	segments	particle morphology	MP
Tombusvirus	TBSV	ssRNA	1	Isometric	p22[1]
Aureusvirus	CLSV	ssRNA	1	isometric	p27[2]
Tymovirus	TYMV	ssRNA	1	isometric	ORF-69[3]
Waikavirus	MCDV	ssRNA	1	isometric	P1[4]
Capillovirus	ASGV	ssRNA	1	filamentous	ORF2[5]
Trichovirus	ACLSV	SsRNA	1	filamentous	50K[6]
Vitivirus	GVA	SsRNA	1	filamentous	ORF3[7]
Tenuivirus	RGSV	SsRNA	4	filamentous	4c[5]
Nucleorhabdovirus	SYNV	SsRNA	1	rhabdo- or bacilliform	Sc4[5]
Sobemovirus	SBMV	SsRNA	1	isometric	P3[8]
Nanovirus	BBTV	SsRNA	6+	isometric	ORF4[9]
Umbravirus	GRV	SsRNA	1	-	ORF4[10]

[a] TBSV, tomato bushy stunt virus; CLSV, cucumber leaf spot virus; TYMV, turnip yellow mosaic virus; MCDV, maize chlorotic dwarf virus; ASGV, apple stem grooving virus; ACLSV, apple chlorotic leaf spot virus; GVA, grapevine virus A; RGSV, rice grassy stunt virus; SYNV, sonchus yellow net virus; SBMV, southern bean mosaic virus; BBTV, banana bunchy top virus; GRV, groundnut rosette virus

Key references: 1, Scholthof et al. (1995); 2, Miller et al. (1997); 3, Bozart et al. (1992); 4, Melcher (2000); 5, Yoshikawa and Takahashi (1988); 6, Yoshikawa et al. (1999); 7, Minafra et al. (1997); 8, Sivakumaran et al. (1998); 9, Burns et al. (1998); 10, Taliansky et al. (1996)

Trans-complementation of cell-to-cell movement

In the preceding sections, we have considered the various mechanisms plant viruses use for cell-to-cell movement and reviewed the role of the viral MPs in this process. It is remarkable that such a large variety of MPs have developed in the course of evolution for a similar function of transferring virus nucleic acid from one cell to an adjacent cell

through plasmodesmata. A limited number of mechanisms for cell-to-cell spread have developed in plant viruses with a diversity of MPs. Various MPs have several qualities in common. Many MPs can bind non-specifically to nucleic acids and have been shown to possess NTPase activity. Furthermore, MPs are targeted to plasmodesmata and modify plasmodesmal structure by increasing plasmodesmal SEL, although the extent in which plasmodesmata are modified differs.

Lack of homology between different MPs

The identification of viral MPs has been accompanied by an intensive search for amino acids conserved between different MPs and between MPs and host proteins that could shed light on the functioning of MPs and establish evolutionary relationships among the MPs. The reference for these homology searches has been the P30 MP of TMV as this was the first protein for which different functions were distinghuished. The results of the complementation studies summarised in the next paragraph, which demonstrated that complementation of cell-to-cell movement can occur between unrelated plant viruses, has further enhanced such an inquiry. However, despite the findings that MPs have several characteristics in common, like targeting to plasmodesmata, the level of amino acid sequence conservation between different MPs is quite low. Cases in which viral MPs show more than 30% amino acid homology to each other are rare, and mostly found if MPs from viruses classified in the same genus are compared. For example, the MPs of the tobamoviruses TMV and GCMV share 37% identical amino acids (Saito et al., 1988), and the homology found between comoviral MPs ranges from 21.8 to 31.8% (Hauldenshield and Palukaitis, 1998). These relatively high percentages of conserved amino acids are usually not found when comparing MPs of viruses belonging to different genera. For example, no homology has been detected when comparing the amino acid sequence of the tubule-forming MPs of caulimo-, como- and nepoviruses (Mushegian, 1994). Similarly, the TMV and RCNMV MPs do not show any obvious sequence homology (Lommel et al., 1988). Despite this low overall conservation, a stretch of 33 amino acids, known as the 30K-superfamily domain, was detected in MPs belonging to 20 different genera, including tobamo-, como-, alfamo- and sobemoviral MPs (Koonin et al., 1991; Mushegian and Koonin, 1993). However, the relevance of this conservation is unclear for several reasons. First, none of these 33 residues is absolutely conserved. The best matches are an aspartic acid residue (known as the D-motif) and a glycine residue (the G-box), which were found in a majority of these MPs (Koonin et al., 1991). Furthermore, the results of various mutational analyses do not give a clear indication about a conserved function of this domain within these MPs (see Kahn et al., 1998, Bertens et al., 2000). Melcher (2000) suggested that the homology

might not be found within the amino acid sequence, but rather on the level of the secondary structure. Computer-assisted analysis of 30K superfamily domains revealed a common structure of a series of β-elements that are flanked by two α helices and suggest that this profile of secondary structure elements have a role in folding of the mature proteins (Melcher, 2000).

Complementation

A surprising feature of many plant viruses is that in many cases a defect in MP function can be complemented by the action of an other MP expressed *in trans* (reviewed in Atabekov and Taliansky, 1990; Atabekov et al. 1999). This has been studied in several ways. Most of the older experiments involve conditions of mixed infections with a movement-defective virus and a helper virus. Later, more direct approaches to study complementation were developed, including the analysis of recombinant viral genomes bearing a heterologous MP gene, the analysis of movement-defective viruses in transgenic plants expressing a viral MP and study of plants co-bombarded with cDNA clones of a movement-defective virus and a helper MP.

Complementation was first described with *ts* mutants of TMV, like Ls1 and Ni2519, which were unable to achieve infection of plants at non-permissive temperatures, but accumulated normally in protoplasts at the non-permissive temperature. The cell-to-cell movement of these *ts* mutants at the non-permissive temperature could be complemented by co-infection with wild-type TMV (Taliansky et al., 1982a,b). Such complementation also occured between different virus species. For example, cucumber green mottle tobamovirus (CGMV) and sunn-hemp mosaic tobamovirus (SHMV) could spread in non-host tobacco plants which were pre-inoculated with TMV (Malyshenko et al., 1989). The complementation of the cell-to-cell movement between tobamoviruses can be understood as the transport function is executed by a single MP with distinct domains for non-specific RNA binding, plasmodesmal targeting and increase of the size exclusion limit of plasmodesmata. Further support for this explanation was found with transgenic plants expressing TMV P30 MP in which TMV mutants defective in the transport function due to mutation of the P30 protein were complemented and the defect in cell-to-cell transport was overcome (Deom et al., 1987).

Complementation is not restricted to tobamoviruses. For example, cell-to-cell spread of a movement-defective dianthovirus RCNMV was rescued in TMV P30 MP-expressing transgenic tobacco plants (Giesman-Cookmeyer et al., 1995). Also this can be easily understood, since RCNMV and TMV have a similar mechanism of cell-to-cell movement, and the RCNMV MP acts in a way similar to the TMV MP. Also a chimeric TMV in which the gene coding for the P30 protein was replaced by the gene of the

RCNMV MP was able to spread systemically in tobacco plants (Giesman-Cookmeyer et al., 1995).

More striking than the above examples was the observation that the TMV Ls1 mutant could also be complemented by PVX (Taliansky et al., 1982a), for the spread of PVX is mediated by the three TGB-coded proteins, and no sequence similarities have been found between the TMV MP and the three PVX TGB proteins. Besides, the viral CP plays an essential role in the mechanism of cell-to-cell movement of PVX. Furthermore, the RNA-binding of the TGB proteins is rather weak and not comparable to the RNA-binding of the P30 protein. The reverse complementation can also occur: TMV MP can functionally replace the TGB proteins of hordei- and beneviruses (Solovyev et al., 1996, Lauber et al., 1998) and cell-to-cell transport of movement-defective PVX can be complemented by the TMV or RCNMV MP supplied by cDNA clones co-bombarded with defective PVX onto *Nicotiana benthamiana* leaves (Morozov et al., 1997). Apparently, the TMV and RCNMV MPs act autonomously in such infections, i.e. function without a need for other viral proteins. Tobamoviral MPs can even complement movement-defective red clover mosaic comovirus (RCMV), a typical example of a virus that spread from cell-to-cell as virus particles, as demonstrated by the ability of SHMV to act as a helper virus for cell-to-cell spread of RCMV RNA1 (Malyshenko et al., 1988). The results of various experiments suggest that *trans*-complementation of cell-to-cell movement is a non-specific process that can occur between viruses with different genome composition, particle morphology or spread from cell-to-cell via different mechanisms (Atabekov and Taliansky, 1990; Atabekov et al. 1999).

Complementation has its limitations. In general complementation, is more effective under conditions of mixed infections of viruses than in experiments involving hybrid viruses or co-bombardement of movement-defective virus and cDNAs supplying MP (Atabekov et al., 1999). For example, BSMV in which the TGB proteins were precisely replaced with the TMV MP, could only spread in hosts susceptible for both BSMV and TMV (Solovyev et al., 1996). Several viruses defective in cell-to-cell movement, including PVX, BSMV and RCMV, could not be complemented by transgenic plants expressing P30 (Atabekov et al., 1999). These results suggest that apparently the presence of the 30K MP is essential but not sufficient for complementation and that some additional TMV or host factors are needed for successfull complementation. An other example illustrating the limitations of complementation is that, while PVX is able to complement the deficiency in cell-to-cell movement of TMV Ls1, the RNA-binding of the PVX CP is specific and probably cannot replace the 30K MP in binding TMV RNA. The MP defective in Ls1 movement probably has retained its RNA-binding capacity that provides for the RNA-binding needed for complementation by PVX.

Complementation involving viruses of other groups may have even more limitations. No complementation was observed in hybrid beneviruses in which the TGB proteins were replaced by the tubule-forming MPs of AMV and GFLV (Lauber et al., 1998) or in cases where defective PVX was co-bombarded with cDNA clones coding for the BMV MP (Morozov et al, 1997). Furthermore, so far it has not been reported that como- or nepoviruses are able to complement defects in transport functions of other viruses, suggesting that their MPs may require specific interactions with other viral proteins, notably their CPs, as suggested by Lekkerkerker et al. (1996) and Belin et al. (1999). Such a requirement may impede the capability of tubule-forming viruses to act as helper viruses in complementation experiments.

In conclusion, it is clear now that the cell-to-cell spread of viruses with defects in their MP can sometimes be complemented by the actions of a second MP, that acts as a helper MP. This finding is quite remarkable, since many details of the infection processes of the two viruses involved need to be coordinated, like the regulation of viral replication and place and timing of MP expression and targeting. In addition, host and other viral factors specifically interacting with the helper MP also influence complementation and complicate the interpretation of such experiments (Atabekov et al., 1999).

Do viruses exploit a host pathway for plasmodesmal transport?

Since viruses are known to exploit host-derived components for their replication (Strauss and Strauss, 1999), one can speculate how virus-specific cell-to-cell movement is, and to what extent plant viruses use an existing plant pathway for the intercellular spread of proteins and nucleic acids for their own purposes. Recently, some plant proteins and nucleic acids have been found to move through plasmodesmata (Lucas et al., 1995; Kuhn et al., 1997). This makes it tempting to speculate that viruses utilise plant mechanisms for plasmodesmal transport of macromolecules for the cell-to-cell spread of their genomes. Unfortunately, so far the mechanisms that plants have developed for cell-to-cell transport of proteins and nucleic acids have not been unravelled, and there is no direct experimental evidence that viruses use a host mechanism for their own purposes. Plant proteins that were shown to traffic through plasmodesmata, like the transcription factor KNOTTED1 (Lucas et al., 1995) and the phloem protein SUT1 (Kuhn et al., 1997) show no homology to viral MPs. Other attempts to find plant proteins with high homology to viral MPs by computer-assisted searches have been largely unsuccessful. The only examples of possible homology are the homology between the closterovirus MP and the family of cellular heat shock proteins (Agranovsky et al., 1991)

and the sequence motifs characteristic for helicases that were detected in TGBp1 proteins (Gorbalenya and Koonin, 1993). Recently Xoconostle-Cázares et al. (1999) characterised a cucumber protein, CmPP16, which has four sequence motifs with the RCNMV MP in common. CmPP16 is localised in the phloem and has the ability to spread from companion cells to neighbouring sieve elements. The protein can bind RNA non-specifically, a feature also found in many viral MPs. Its function might be transport of RNA molecules through the phloem as part of a plant information highway (Xoconostle-Cázares et al., 1999). These different observations do not give a clear picture of plasmodesmal transport in plants.

It has also been proposed that cell-to-cell movement of macromolecules through plasmodesmata resembles transport of proteins and nucleic acids through nuclear pores (Lucas and Wolf 1993, Citovsky and Zambryski, 1993, Ghoshroy et al., 1997). Both plasmodesmata and nuclear pores are complex proteinaceous structures involved in bidirectional traffic of macromolecules (Ghoshroy et al., 1993; Davis, 1995). Although small molecules can freely diffuse across nuclear pores, large components as proteins and nucleic acids are actively transported across the nuclear pore (Davis, 1995, Forbes, 1992). During this process the diameter of the nuclear pore channel increases, a process comparable to the increase in plasmodesmal SEL. The transport process is energy-dependent and involves recognition of specific nuclear localisation signals or nuclear export signals on the proteins involved (Görlich, 1997). For transport through nuclear pores, nucleic acids form complexes with specific proteins. These complexes are subsequently unfolded during the transport process.

The details of the mechanisms by which proteins are targeted to and translocated through plasmodesmata are still unknown. It has been speculated that host proteins like KNOTTED1 and viral MPs contain a plasmodesmata localisation signal (PLS) (Citovsky, 1999). Attempts to characterise this PLS have not been successful until now. The putative PLS may interact with a cytoplasmically localised receptor, which guides the transported protein to plasmodesmata. Alternatively, elements of the cytoskeleton and/or endoplasmic reticulum might be involved in intercellular transport of proteins to plasmodesmata, where a PLS would be recognised by a plasmodesmata-localised receptor. Kragler et al. (1998) postulated that proteins like KNOTTED1 and the TMV MP undergo a conformational change before entering an extended plasmodesmal channel. Both host proteins like KNOTTED1 and viral MPs seem to use the same plasmodesmal receptor (Kragler et al., 1998). The identity of the putative plasmodesmal receptor is still unknown, although the cell-wall associated enzyme pectin methylesterase is a good candidate for such a role (Citovsky, 1999; Dorokhov et al., 1999; Chen et al., 2000).

Concluding remarks

In this review, we have given an overview of plant virus cell-to-cell movement and the roles of a specialised class of viral proteins, the MPs, in this process. For most viral genera MPs have now been isolated, but for many of these, details about the mechanisms that are used for cell-to-cell movement are still scarce. Despite this lack of information, the most important features of the different mechanisms are known and it is unlikely that the characterisation of other MPs will lead to totally new concepts for cell-to-cell movement. Probably, the newly identified MPs will either use one of the two major mechanisms, movement via tubules as virions or movement as viral nucleoprotein particles. In the coming years, the research will extend to the identification and characterisation of host factors that interact with viral MPs, like the recently characterised pectin methylesterase and the DNA J chaperonin that was found to interact with the TSWV MP (Soellick et al., 2000). Other screens have identified genetic loci that influence cell-to-cell movement but not replication of plant viruses. For example, Callaway et al. (2000) identified three loci in the genome of *Arabidopsis thaliana* that influence the susceptibility of CaMV MP mutants and Yoshi et al. (1998a,b) identified two *Arabidopsis* loci, known as *cum*-1 and *cum*-2, that affect the cell-to-cell movement of CMV and TMV. The characterisation of these and other loci will be easier in the near future, since the complete sequence of the *Arabidopsis* genome will be available at the end of this year (Meyerowitz, 1999).

Recently, progress has also been made in the understanding of the long-distance movement of viruses through the vascular system of the plant (reviewed in Carrington et al., 1996, Santa Cruz, 1999). There are now clear indications that long-distance movement may require viral and host functions distinct from those involved in cell-to-cell movement. For example, several viruses that can spread from cell-to-cell in the absence of CP need this protein for long-distance movement (Vaewhongs and Lommel, 1995). Most viruses are thought to spread through the phloem as virions. An exception may be formed by the umbraviruses, a class of plant viruses that do not code for a CP. Instead, the product of ORF3, that has nucleic acid-binding properties, might bind to the viral RNA and protect it from degradation in the phloem (Ryabov et al., 1999). A yet unanswered question is how viruses enter and leave the vasculate. It has been established that the complex of companion cells and sieve elements represents the major barrier for entry of plant viruses into the phloem and also acts as a traffic checkpoint for host components that enter and exit the phloem (see Boevink et al., 1999).

Chapter 2

MUTATIONAL ANALYSIS OF THE COWPEA MOSAIC VIRUS MOVEMENT PROTEIN

Abstract

Cowpea mosaic virus (CPMV) moves from cell-to-cell in a virion form through tubular structures that are assembled in modified plasmodesmata. Similar tubular structures are formed on the surface of protoplasts inoculated with CPMV. The RNA 2-encoded movement protein (MP) is responsible for the induction and formation of these structures. To define functional domains of the MP, an alanine-substitution mutagenesis was performed on eight positions in the MP, including two conserved sequence motifs, the LPL- and D-motifs. Results show that these two conserved motifs as well as the central region of the MP are essential for cell-to-cell movement. Several viruses carrying mutations in the N-terminal or C-terminal parts of their MP retained infectivity on cowpea plants. Co-expression studies revealed that mutant MPs did not interfere with the activity of wild-type MP, and could not mutually complement their defects.

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Introduction

For successful systemic infection of a plant, a virus must be able to replicate in and spread throughout the plant. The spread of a plant virus involves first local movement from cell-to-cell and then, if it reaches the vascular system, long-distance movement that brings about systemic infection of the plant. During cell-to-cell spread, plant viruses have to pass the barrier formed by the rigid cell wall. This obstacle is overcome with the aid of a special class of proteins called movement proteins (MPs). These MPs can alter the structure of plasmodesmata, small intercellular channels that are normally used by the plant for intercellular communication (McLean et al., 1997). Currently, evidence is accumulating that several mechanisms of cell-to-cell movement exist and that each involves a specific modification of plasmodesmata (reviewed in Carrington, 1996; Ghoshroy et al., 1997; Lazarowitz and Beachy, 1999).

Cowpea mosaic virus (CPMV) is a small icosahedral virus with a genome that consists of two RNA molecules, called RNA1 and RNA2 (for a review see Goldbach and Wellink, 1996). RNA1 codes for proteins involved in replication of the viral RNA. RNA2 codes for two capsid proteins (CP) denoted L and S, and two C-terminally overlapping proteins (the 58kDa and 48kDa MP respectively). Both the MP and the CPs are necessary for cell-to-cell movement (Wellink and Van Kammen, 1989, Verver et al., 1998). The 58K protein is thought to be involved in replication of RNA2 (Van Bokhoven et al., 1993b).

In the cell walls of CPMV-infected plant cells long tubular structures filled with virus-like particles have been found in modified plasmodesmata (Van Lent et al., 1990). Immunogold labelling using antisera raised against the 58K/MP proteins showed that the 58K and/or the MP is localised in these tubules. Similar tubular structures are found on the surface of CPMV-infected protoplasts, where they extend into the culture medium (Van Lent et al., 1991). Mutants that fail to produce the MP do not form these tubular structures, suggesting that the MP is essential for this process (Kasteel et al., 1993). Upon transient expression of the MP alone in protoplasts, tubular structures are also produced, demonstrating that the MP is the only viral protein necessary for the formation of the tubular structures. Surprisingly, tubules were also formed in protoplasts isolated from non-host plants upon transient expression of the MP protein (Wellink et al., 1993). Even transient expression of the MP in insect cells using a baculovirus expression system led to the formation of MP-derived tubular structures, suggesting that plant-specific proteins are not absolutely necessary for tubule formation (Kasteel et al., 1996). If host factors are involved in tubule formation they should be of a very conserved nature.

To identify functional domains in the MP we conducted a mutational analysis of the MP. Previously, we analysed the effects of random insertion and deletion mutations

on the functioning of the MP (Wellink and Van Kammen, 1989; Lekkerkerker et al, 1996). In this report, we extended this analysis using alanine-substitution mutagenesis. With this method, the chance of disturbing the tertiary structure of the protein involved is kept to a minimum (Cunningham and Wells, 1989). Because of the relatively low sequence homology between different MPs, we focused our mutational analysis on residues conserved between distinct comovirus MPs. The properties of the different alanine-substitution mutants were then analysed in protoplasts and plants.

Results

Alanine-substitution mutants

To identify functional domains of the CPMV MP a series of alanine-substitution mutants was constructed. Because the overall similarity between comoviral MPs is rather low (varies from 21.1% to 31.8% (Haudenshield and Palukaitis, 1998)), it is conceivable that the conserved regions have important roles in the normal functioning of the MP. To compare the sequences of the different MPs, first an alignment from the complete amino acid sequences of six comoviral MPs was made (Figure 1). Most of the conserved residues can be found in the central region of the CPMV MP, between residues 115 and 248 (Chen and Bruening, 1992; Figure 1). A part of this area, residues 116 to 148, corresponds to the 30K-superfamily domain, which has been found in MPs of at least 17 plant virus genera (Mushegian and Koonin, 1993). The main feature of this domain is an almost absolutely conserved aspartic acid residue, named the D-motif (corresponding to amino acid 143 of the CPMV MP). In the N-terminal part of the CPMV MP a second conserved sequence motif (the LPL-motif) is present that has been found in MPs of viruses belonging to five different genera (Koonin et al., 1991). In addition to the conserved central region and LPL-motif, only small areas of conserved residues were found (Figure 1). Computer-assisted analysis of the MP sequences predicts that the secondary structures of N-terminal and central regions of the comoviral MPs are similar (Chen and Bruening, 1992 and data not shown). The central regions display a typical alternation of hydrophobic and hydrophilic regions. The D-motif is localised in a hydrophobic region.

Alanine-substitution mutations were created by site-directed mutagenesis using specific oligonucleotides, into an infectious full-length clone of RNA2, pTM1G. As a result, in each mutant MP two adjacent amino acids were changed into alanine residues (Figure 1). Mutations in MAM-1 (residues 9 and 10) and MAM-3 (residues 103 and 104) were located in conserved motifs in the N-terminal part of comoviral MPs.

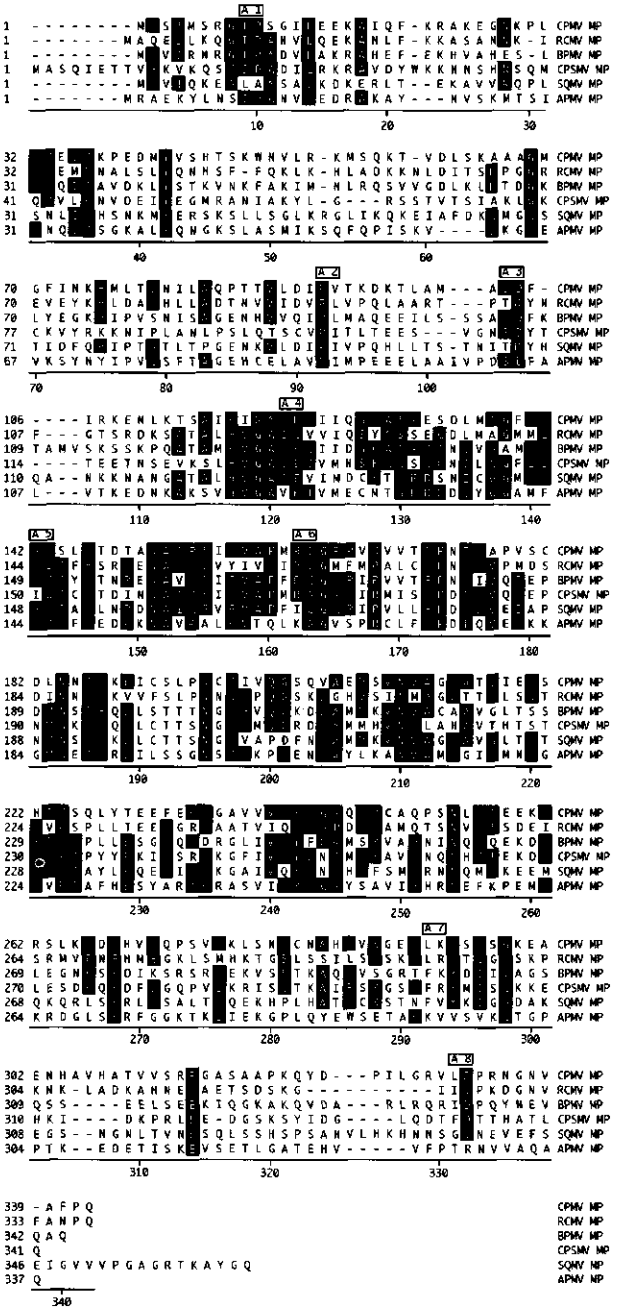

Figure 1: Alignment of comoviral MPs. The positions of the alanine-substitution mutations in the CPMV MP are indicated as blocks MAM1 to MAM8 above the sequences. Identical amino acids present in at least 4 of the 6 sequences are in black. The numbering below the alignment represents that of the CPMV MP. EMBL accession numbers for the RNA 2-derived polyproteins of the different viruses aligned are: APMV, L16239; BPMV, M62738; CPMV, X00729; CPSMV, M83309; RCMV, M14913 and SQMV-K, AF059533. The alignment was generated using the Clustal-W program within the Lasergene Navigator

software package (DNASTAR, Inc, Madison, WI) with a PAM 250 residue weight table followed by manual manipulation of the output in the region around the LPL-motif and the C-terminus.

The mutation in MAM-2 (residues 92 and 93) was located in the LPL motif and in MAM-5 (residues 142 and 143) the D-motif was changed. Mutations in MAM-5 and MAM-4 (residues 121 and 122) were localised in hydrophobic areas in the 30K-superfamily domain. In MAM-6 (residues 162 and 163) the mutation was placed in a hydrophilic area of the conserved central region of the CPMV MP. The mutation in MAM-7 (residues 292 and 293) was located around the C-terminal border of the tubule forming domain of the MP and MAM-8 (residues 331 and 332) was located in the highly divergent C-terminal part of the MP, a part thought to be involved in the interaction between movement protein and virus particles (Lekkerkerker et al., 1996).

Effects on replication

The effects of the alanine-substitution mutations on RNA2-replication and tubule formation were studied through the inoculation of cowpea protoplasts with *in vitro* generated transcripts. In all cases protoplasts were inoculated with transcripts of wild-type RNA1 and wild-type or mutant RNA2 (hereafter we mention only the RNA2(s) used). Because the MP and 58K proteins are C-coterminal, mutations in the MP also lead to mutations in the 58K protein. Because the 58K protein is thought to be involved in replication of RNA2, it was essential to investigate if the mutations introduced into the MP had any effects on replication of RNA2. Because viral replication and translation are tightly linked, immunofluorescence analysis and Western blotting of inoculated protoplasts can be used as a measure for viral replication (Van Bokhoven et al., 1993b). The percentage of virus-infected protoplasts detected 48 hrs post inoculation was similar between mutants and wild-type (Table 1), suggesting that the introduced mutations did not affect replication of the RNA2s. This was confirmed by Western blot analysis of inoculated protoplasts (data not shown). Hence, we concluded that the mutations introduced into the 58K and MP proteins did not have a negative influence on replication of RNA2. This is consistent with earlier findings that the replication of RNA2 molecules that have large deletions within the MP coding region or that contained small insertions or point mutations is not significantly affected (Van Bokhoven et al., 1993b; Lekkerkerker et al., 1996).

Tabel 1: Characteristics of CPMV MP alanine-substitution mutants in protoplasts and plants

mutant	percentage of protoplasts staining with anti-CPMV	tubules on protoplasts[a]	infectivity on cowpea plants[b]	Mean diameter of lesions that developed on Pinto's[c] (mm)
wt	9	+	+	2.20 ± 0.67
AM1	10	+	+	0.20 ± 0.11
AM2	9	+/-	-	NT
AM3	8	+	+	0.18 ± 0.11
AM4	9	-	-	NT
AM5	9	-	-	NT
AM6	8	-	-	NT
AM7	10	+	+	1.41 ± 0.75
AM8	10	+	+	0.49 ± 0.27

[a]) ability of the MP to make tubular structures on rotoplasts (48 hours post inoculation): (+) tubular structures regularly detectable on the surface of protoplasts; (+/-) very rarely tubules detected; (-) no tubules detected;

[b]) cowpea plants were inoculated with protoplast extracts: (+) symptoms detectable on inoculated and higher leaves and RT-PCR product found; (-) no symptoms detectable on inoculated and higher leaves and no RT-PCR product found

[c]) mean size of lesions that developed on the inoculated leaves measured 8 days post inoculation (diameter in mm ± standard deviation of 50 measurements). NT = not tested.

Effects on tubule formation and subcellular localisation

To investigate the effects of the various mutations on tubule formation, inoculated protoplasts were labelled with antisera raised against the 58K/MP proteins. Only the 3 N-terminal mutants MAM1, MAM2 and MAM3 and the 2 C-terminal mutants MAM7 and MAM8 were able to induce the formation of tubules, as observed 48 hr after inoculation (Table 1). Mutant MAM2, having a mutation in the LPL-motif, was severely disturbed in tubule formation. In only 3 out of 20 experiments were tubular structures detected in protoplasts inoculated with this mutant. In each of these experiments, only a small subset of the inoculated protoplasts contained tubular structures. Mutants MAM4, MAM5 and MAM6 did not form tubular structures. Microscopic observation of individual protoplasts infected with these mutants suggested that the MP had a cytoplasmic localisation (data not shown). To investigate the subcellular localisation of these mutants in more detail, protoplast extracts were fractionated and analysed by SDS-PAGE. In protoplasts inoculated with wild-type RNA2 transcripts, most of the 58K protein is present in the cytoplasmic fraction (S30) and the majority of the MP is present in the membrane fraction (P30) (Rezelman et al., 1989; Figure 2, lanes 6 and 7). This ratio changed for mutants MAM2, MAM4, MAM5 and, to a lesser extent, MAM6 (Figure 2). Clearly a higher portion of the MP was present in the cytoplasmic fraction whereas the proportion of the 58K protein present in the P30 and S30 fractions did not change, suggesting that the intracellular distribution of the mutant MPs was altered.

It was therefore concluded that MPs with alterations in their central region have become deficient in tubule formation in protoplasts. This might be due to a less efficient targeting of the MP to the plasma membrane of the cell. The MAM2 mutant seems to take an intermediate position as most of this mutant MP accumulated in the cytoplasm although some tubular structures were still formed.

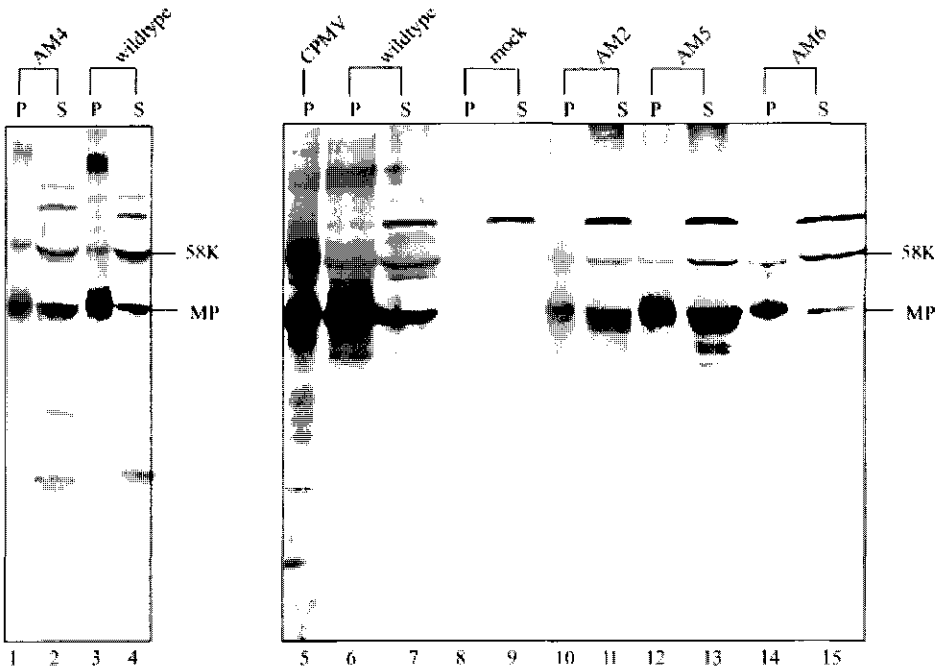

Figure 2: Western blot analysis of inoculated cowpea protoplasts. Protoplasts were inoculated with RNA transcripts and extracted 48 hrs later. Extracts were centrifuged to obtain S30 and P30 fractions. Equal amounts of protoplast samples were separated on a 12.5% SDS-PAGE and blotted onto nitrocellulose. The blot was treated with anti-58K/MP serum. Lanes 1, 3, 6, 8, 10, 12 and 14 represent P30 fraction; lanes 2, 4, 7, 9, 11, 13 and 15 represent S30 fractions. Lane 5: membrane fraction obtained from CPMV-infected cowpea leaves; other lanes represent fractions of inoculated protoplasts. Positions of the 58K and MP are indicated on the right side of the blot.

Effects on virus spread

To test the ability of viruses carrying a mutation in their MP to spread in plants, cowpea plants were inoculated with a mixture of transcripts of wild-type or mutant RNA2 or with extracts of inoculated protoplasts. Infection was assayed by the appearance of symptoms and by RT-PCR analysis using RNA2-specific primers. Mosaic symptoms were detectable on inoculated leaves of plants inoculated with wild-type RNA2 after 3-4 days. Symptoms appeared 2-3 days later on plants inoculated with transcripts of pTMAM1, pTMAM3, pTMAM7 or pTMAM8. Plants inoculated with wild-type RNA2

transcripts developed symptoms 5-6 days post inoculation on higher leaves. Symptom development was delayed on plants inoculated with mutant viruses: symptoms became visible 4-5 days later than on plants inoculated with wild-type RNA2. The time of symptom appearance was comparable for the different mutants. There were no gross differences between the severity of symptoms induced on plants inoculated with mutant viruses: mildest symptoms were found on plants inoculated with MAM8 and symptoms slightly more severe than wild-type were found on plants inoculated with MAM1, MAM3 and MAM7 (data not shown). No symptoms were detectable on leaves of plants inoculated with MAM2, although this mutant was able to form tubular structures in protoplasts.

Plants inoculated with the three other mutants, MAM4, MAM5 and MAM6, which were not able to form tubular structures in protoplasts, did not develop any symptoms on primary or higher leaves up to 14 days after inoculation.

CPMV RNA2-specific RT-PCR products could be amplified from total RNA of primary and higher leaves of plants inoculated with wild-type transcripts and transcripts of mutants pTMAM1, pTMAM3, pTMAM7 or pTMAM8. As in all mutants, together with the mutation, a new restriction site was introduced, retention of the mutation could be easily verified by restriction enzyme analysis. In all cases this restriction site was retained, showing that the mutation was stable (data not shown). No RT-PCR products could be detected in plantsinoculated with transcripts of pTMAM2, pTMAM4, pTMAM5 or pTMAM6 or in noninoculated plants, indicating that these mutants were defective in cell-to-cell movement (data not shown). To investigate whether cell-to-cell movement was completely blocked, or if limited cell-to-cell movement occurred, mutants MAM2, MAM4, MAM5 or MAM6 were introduced into plasmid pTMGFP. This construct contains a wild-type CPMV RNA2 sequence supplemented with green fluorescent protein (GFP) between the MP and L genes as a reporter (Verver et al., 1998). Cowpea plants were inoculated with extracts of protoplasts inoculated with transcripts of pTMGFP, pTMAM2GFP, pTMAM4GFP, pTMAM5GFP or pTMAM6GFP. MGFP was highly infectious in cowpea plants and clusters of green fluorescent cells could be readily identified by illuminating the plants with a hand-hold UV-source (Figure 3A shows a confocal microscopic image of such an infection focus). On plants inoculated with transcripts of pTMAM2GFP, pTMAM4GFP, pTMAM5GFP or pTMAM6GFP only single fluorescent cells could be detected (Figure 3B and data not shown). These results confirmed that mutants MAM2, MAM4, MAM5 and MAM6 were able to replicate in plant cells but that the lack of symptoms and detectable RT-PCR products was due to absence of cell-to-cell movement.

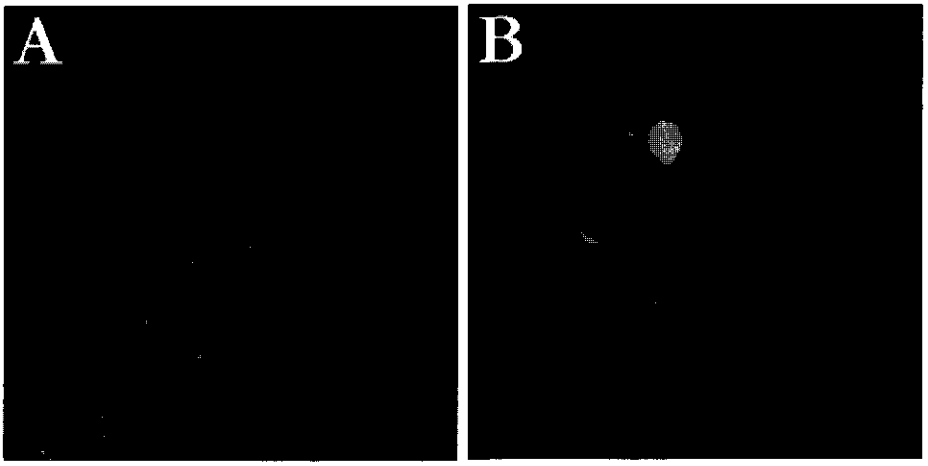

Figure 3: Confocal laser scanning microscope image of epidermal cells of a cowpea leaf inoculated with transcripts of RNA1 and either pTMGFP (A) or pTMAM2GFP (B) at 4 days post inoculation.

From these analyses we conclude that MPs with mutations in the central region or the LPL-motif are not able to support virus cell-to-cell movement *in planta*. Viruses containing mutations in two motifs that are only conserved between different comoviral MPs and in the C-terminal region of the MP are still able to infect plants, suggesting that these mutations are located in regions that have mainly a structural role.

Analysis on *Phaseolus vulgaris* cv. Pinto

The effect of the mutations in the MP was also tested on a local lesion host, i.e. *Phaseolus vulgaris* cv. Pinto (Van Kammen and De Jager, 1978). Leaf extracts were prepared from higher leaves of infected cowpea plants and inoculated onto 8 day old Pinto leaves. Each of the viruses tested produced lesions on Pinto. On beans inoculated with wild-type CPMV, lesions were first visible by 3 days post-inoculation and developed into lesions with a mean diameter of 2.2 mm 8 days post-inoculation (Table 1). The average diameter of the lesions which developed on leaves inoculated with mutants MAM1, MAM3, MAM7 and MAM8 was smaller than lesion which developed on plants inoculated with wild-type CPMV (Table 1). The most severe effects on lesion development were observed with mutants MAM1 and MAM3; the lesions induced on plants inoculated with these mutants had a mean diameter approximately 10% of wild-type lesions. Mutants MAM7 and MAM8 had less drastic effects on lesion development, lesions induced by these mutants were 22-64% smaller than lesions formed by wild-type CPMV. So, in general, mutants with an alteration in the N-terminal part of the MP

had a more severe effect on lesion development than mutants with changes in their C-terminal part.

Possible complementation or interference between mutant and wild-type MPs

Next, the ability of the mutant MPs to interfere with functioning of the wild-type MP in cowpea plants was tested. For this, protoplasts were inoculated with equal amounts of transcripts of pTMGFPΔCP (which codes for wild-type 58K and MP proteins and GFP) and pTMAM2, pTMAM4, pTMAM5 or pTMAM6. Protoplast extracts were used to inoculate primary leaves of cowpea plants. As a control, plants were inoculated with extracts of protoplasts inoculated with CPMV-TRI, consisting of MGFPΔCP and MΔ48 (which lacks the MP) (Verver et al., 1998). On plants inoculated with CPMV-TRI fluorescent spots developed 3-4 days after inoculation (Verver et al., 1998). Green fluorescent spots of a similar size became apparent 3-4 days after inoculation on plants inoculated with the alanine-substitution mutants and MGFPΔCP RNA, suggesting that the presence of the mutant MPs did not interfere at all with the cell-to-cell movement. To verify that the RNA coding for the mutated MP was still present in these leaves, total RNA was extracted and amplified by RT-PCR using primers M512+ and M1952-. As shown in figure 4, PCR fragments were detected in all these samples, and restriction analysis of the fragments confirmed the presence of the mutations. In a second RT-PCR reaction we also confirmed the presence of the MGFPΔCP RNA in these plants (data not shown).

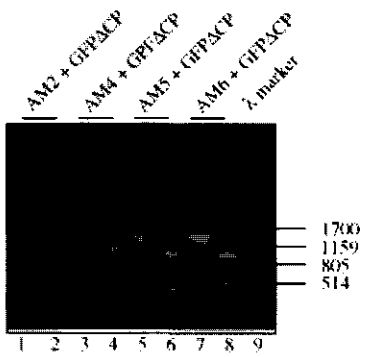

Figure 4: 0.8% agarose gel of RT-PCR fragments amplified from RNA isolated from plants inoculated with extracts of protoplasts inoculated with transcripts of RNA1, pTMGFPΔCP and pTMAM2 (lanes 1 and 2), pTMAM4 (lanes 3 and 4), pTMAM5 (lanes 5 and 6) and pTMAM6 (lanes 7 and 8). DNA fragments were amplified with primers M512+ and M1641-. The presence of the mutation was confirmed by digestion of RT-PCR fragments with the restriction enzymes unique for each mutation. Lanes 1, 3, 5 and 7 represent undigested PCR fragments, lanes 2, 4, 6 and 8 represent digested PCR fragments. Some of the sizes of the λ-marker (lane 9) are indicated at the right side of the gel.

The previously studied mutant MΔXB (which codes for a MP lacking 18 amino acids in its C-terminal part) has been shown to produce tubular structures in protoplasts (Lekkerkerker et al., 1996). However, these tubular structures do not contain virus-like particles and therefore it has been proposed that this mutant MP is no longer able to interact with virus particles. Several of the currently studied mutants are blocked in tubule formation. To investigate if these two functions need to be expressed by a single MP molecule, protoplasts were inoculated with MΔXB and either MAM2GFP or MAM4GFP transcripts. Next, cowpea plants were inoculated with extracts of these protoplasts. In contrast to plants inoculated with MΔ48 and MGFPΔCP, no large fluorescent spots were detectable after up to 14 days after inoculation. Only single fluorescent cells were detectable 3-5 days after inoculation, similar to the cells shown in figure 3B (data not shown). This result suggests that these mutant MPs cannot mutually complement their defects.

Discussion

In this study we have used alanine-substitution mutagenesis in an effort to identify distinct functional domains of the CPMV MP. Mutations in the central part of the MP blocked the ability of the MP to induce the formation of tubules in protoplasts. When these mutations were introduced into a virus which is able to express GFP, only single fluorescent cells were detectable on inoculated leaves. This shows that these mutations completely blocked cell-to-cell movement. Immunofluorescence and Western blot analysis suggested that some of these mutants might be disturbed in targeting of the MP to the plasma membrane. Most viruses containing mutations in the N-terminal part of the MP and all viruses carrying mutations in the C-terminal part of the MP were infectious on cowpea plants. Between these mutants there were no large differences in infectivity and symptom development. However, the mutant viruses behaved differently after inoculation onto the local lesion host *Phaseolus vulgaris* cv. Pinto. In particular viruses containing mutations in the N-terminal half of the MP formed lesions in Pinto's that were greatly reduced in size. The altered responses of Pinto's to the different mutant viruses could be due to a reduction in cell-cell movement of the mutant viruses. On the other hand, it is also possible that the Pinto's showed an enhanced response to these mutant viruses. When taking into account that the responses of the Pinto's to the mutants is more drastic than the response of the cowpea plants, the latter suggestion seems more likely.

Sequence comparison of comoviral MPs showed that the regions with the highest homology are found in the central part (Fig. 1). Inside this region, a 33-amino acid long domain has some homology with regions in the MPs of viruses belonging to 17 genera (denoted the 30K superfamily domain). Within this 33 residues domain, only one amino acid residue (the D-motif) is almost absolutely conserved (Koonin et al., 1991, Mushegian and Koonin, 1993). Surprisingly, viruses of these 17 genera employ different mechanisms of cell-to-cell movement Carrington et al., 1996). Therefore, the 30K superfamily domain may have a function conserved between these mechanisms, like intracellular targeting to, or modification of, plasmodesmata. The central region had a quite remarkable alternation of hydrophobic and hydrophilic residues that is also conserved among MPs of these groups. All CPMV MP mutants that had mutations in this central region were not able to form tubular structures in protoplasts and immunoflorescence analysis and western blotting suggested that the intracellular distribution of these mutant MPs might have been altered. Mutations have also been made in the 30K superfamily region of tobacco mosaic virus (TMV) and cauliflower mosaic virus (CaMV) MPs. In most of these cases, the altered MPs no longer supported cell-to-cell movement (Thomas et al., 1995; Kahn et al., 1998). The fluorescence observed in protoplasts inoculated with TMV MP-GFP fusion mutants was completely diffuse, suggesting that, like for the CPMV mutants with alterations in the 30K superfamily domain, intracellular targeting was affected (Thomas et al., 1995).

Besides the central region only small sequence motifs are conserved among comovirus MPs. Only one other sequence motif, the LPL motif, is present in MPs outside the comovirus genus. A CPMV MP carrying a mutation in the LPL motif (mutant MAM2) is no longer functional in plants. In protoplasts inoculated with MAM2, occasionally tubular structures can be found. Western blot analysis suggests that most of the MAM2 protein resides inside the cytoplasm. The MAM2 phenotype could be explained by assuming that tubule formation occurs exclusively at the cell membrane while the targeting of the MAM2 MP to this membrane is severely affected but not completely blocked. In the rare occasions when sufficient amounts of MP are present in the cell membrane, tubular structures may be formed since the function of a putative tubule-elongation domain is not affected. This distinguishes MAM2 from the three mutants with alterations in the central region for which targeting to the cell membrane might be completely blocked. A mutation in the LPL motif of the CaMV MP also blocks normal functioning (Thomas and Maule, 1995). As MAM2 is severely disturbed in tubule formation, it might be interesting to know whether this CaMV mutant is able to make tubular structures, as does the wild-type MP of CaMV (Perbal et al., 1993).

Surprisingly, none of the defective alanine-substitution MPs interfered with the normal functioning of wild-type MP during mixed infections. So far we have not been able to

determine whether the mutant MPs interact with the wild-type MP to form hybrid tubules, or that, due to a different intracellular localisation, interactions between wild-type and mutant MP do not take place. Mutants with a defect in tubule formation could not be complemented by a mutant that is able to make tubular structures but seems to be unable to interact with virus particles. Apparently both functions need to be present on one MP molecule. Recently it was shown that intramolecular complementation between mutant TMV MPs is likewise not possible (Kahn et al., 1998).

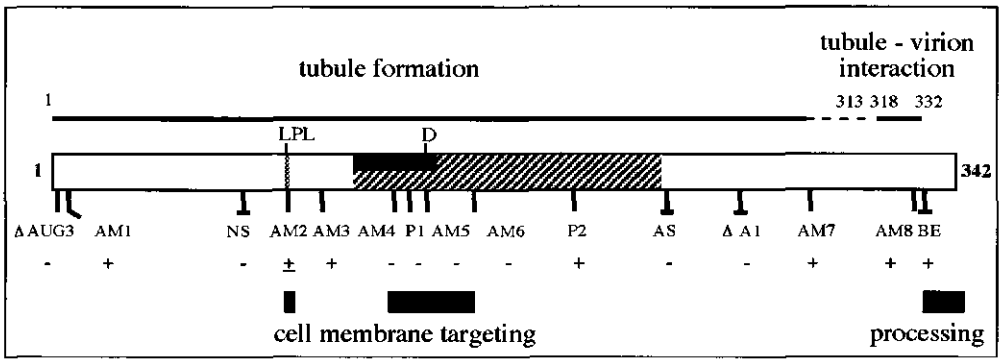

Figure 5: Overview of the structure of and the mutations created in the CPMV MP. The MP is shown as an thin black bar. The LPL-motif is shown as a grey bar. The conserved central region is indicated as a hatched area; the 30K superfamily domain is shown in black. The position of the D-motif is shown above the MP-bar. Positions of alanine-substitution and point mutations (|) and small insertion/deletion mutations (⊥) are indicated below the MP. The characteristics of the MP mutants are shown between brackets (+ able to make tubular structures in protoplasts and to infect plants; ± able to make tubular structures in protoplasts but unable to infect plants; - not able to make tubular structures in protoplasts and not able to infect plants). Regions involved in a certain function are indicated with bars above and below the MP. The C-terminus of the tubule forming domain is located between amino acids 285 and 313 (Lekkerkerker at al., 1996).

An overview of the effects of point and insertional mutations in the CPMV MP on tubule formation and spread in plants as found in this and earlier work is given in figure 5. So far all mutations that have been introduced in the 30K superfamily region of the MP lead to a block in tubule formation. These include the three mutants described in this paper, and mutant P1 (in which the serine residue at position 127 is changed into an isoleucine) (Wellink, unpublished results). Results indicate that this region may have a function in cell membrane targeting. Within the remaining part of the domain required for tubule formation, small alterations of the wild-type sequence do not always block tubule formation, as shown for mutants MAM1, MAM3 and P2. Mutant P2 (in which the glycine residue at position 201 is changed into a proline residue) is able to form tubular structures in protoplasts and is infectious in plants (Wellink, unpublished results). Small mutations generated in the C-terminus of the MP have no effects on tubule formation.

Analysis of the MΔXB mutant suggested that the C-terminus is involved in an interaction between MP and virus particles (Lekkerkerker et al., 1996). It is however remarkable that other mutations generated in this area so far have only minor effects on infectivity and thus seem not to disturb this interaction. Since tubule formation is not blocked for mutant MAM7, the domain required for tubule formation actually may end around amino acid 292 but this will have to be confirmed by constructing C-terminal deletion mutants. From work focused on the development of CPMV as a vector for the *in planta* expression of foreign proteins it has become clear that amino acids 335 to 342 are sufficient for efficient cleavage between MP and L (Gopinath et al., 2000). It is at present unclear if these 7 amino acids have an additional role during cell-to-cell movement of CPMV. The presence of putative other functional domains in the MP (e.g. domains involved in plasmodesmata modification, (homo)polymerisation, NTP-binding and long distance movement) will be subject of further investigations.

Materials and methods

Construction of alanine-substitution mutants

Standard DNA and RNA techniques used in this research were essentially carried out as described by Sambrook et al. (1989). Plasmids were purified after isolation by Qiagen miniprep spin columns (Qiagen). All enzymes were purchased from Life Technologies or Boehringer Mannheim. Plasmids were grown in *Escherichia coli* strain DH5α. All mutants were constructed in pTM1G that contains a full-length cDNA clone of the CPMV RNA2 sequence downstream from a T7 promoter from which infectious RNA2 transcripts can be generated (Eggen et al., 1989). Mutants pTMAM1 to pTMAM8 were created by oligo-directed site-specific mutagenesis according to Kunkel (1985) using following oligonucleotides: 5'-AATTCCTGAAGCGGCGCCACGGCTC-3' (MAM1), 5'-CTTTGTGGG-CGATATCCAAGAC-3' (MAM2), 5'-CGAATAAAAGCCGCGGCCATCC-3' (MAM3), 5'-GGATAATAATCGCTGCAGCTCGAATG-3' (MAM4), 5'-GTGTAAGAAG-CTGCAGAAGC-3' (MAM5), 5'-GGTCTTCCTGCAGCCATTGGAGC-3' (MAM6), 5'-GAA-ATAGAGTATCGCCTTCTCC-3' (MAM7) and 5'-CCATTTCGTGGCGCCGCC-ACCCGTCCC-3' (MAM8). Each mutant contained a restriction enzyme site (underlined) for easy identification. Mutagenesis resulted in the substitution of two consecutive amino acid residues into alanines. Single stranded DNA for mutagenesis was prepared from M13mp19 containing fragments 1-1504 or 1192-2296 of RNA2 cultured in *Escherichia coli* strain CJ236.

Plasmid pTMGFP contains the complete RNA2 cDNA with the coding sequence of GFP inserted between the MP and CP genes (Verver et al., 1998). The alanine-substitution mutants were introduced into pTMGFP by exchanging *Bgl*II/*Afl*II fragments with pTMGFP to obtain pTMAM2GFP, pTMAM4GFP, pTMAM5GFP and pTMAM6GFP. The construction of plasmids pTMGFPΔCP and pTMΔ48 has been described before (Verver et al., 1998). Plasmid MΔXB has been described earlier (Lekkerkerker et al., 1996).

In vitro transcription and inoculation of protoplasts and plants

Transcripts were synthesised as described before (Van Bokhoven et al., 1993a), except that DTT was included in each transcription reaction to a final concentration of 10 mM. The amount and fidelity of the transcribed RNAs was checked on agarose gels. Cowpea (*Vigna unguiculata*) cv. California Blackeye and beans (*Phaseolus vulgaris* cv. Pinto) were grown in growth chambers (16-hr light/8-hr dark periods at 25 °C). Mesophyll protoplasts were isolated out of primary leaves of 9-10 days old cowpea. 10^6 protoplasts were inoculated with 1 μg each of RNA1 and RNA2 using the polyethylene glycol method as described (Van Bokhoven et al., 1993a). Protoplasts were incubated under continuous illumination at 25 °C. After 48 hr an aliquot of the protoplast sample was used for immunofluorescence while the remainder was collected by centrifugation at 3,000 rpm and stored at -80 °C until further use.

Transcription reactions or protoplast extracts were applied manually onto carborundum-dusted upper surfaces of primary leaves of 7-8 day old cowpea plants. Extracts of protoplast samples were prepared by resuspension of the protoplast pellet in 100 μl phosphate buffered saline (made according to Sambrook et al., 1989). This suspension was homogenised by straining through a 0.45 mm syringe for 10-15 times. Inoculated plants were checked daily for the appearance of symptoms. Inoculated and higher leaves were collected at various times after inoculation and stored at -80 °C until further use. Plants inoculated with transcripts of pTMGFP-derived constructs were checked daily for fluorescent cells using a longwave handhold UV-lamp (B100AB; Ultraviolet Products) and/or a Nikon Optiphot 2 microscope, using the UV1A filter set (excitation filter 365/10 nm). Photographs were taken with a Zeiss LSM510 confocal microscope.

Leaves of 7-8 day old Pinto beans were inoculated with extracts from leaves of cowpea plants. Extracts were applied onto carborundum-dusted upper surfaces of Pinto leaves. The leaves were checked daily for the presence of lesions. After 6 to 10 days parts of the Pinto leaves were boiled for 10 min in ethanol to remove the chlorophyll and washed for several hrs in 70% ethanol and water. The air in the leaves was removed under vacuum and the leaves were mounted on microscopic slides covered with 70% glycerol. Samples were investigated under a Nikon SMZ-U binocular.

Western blotting

For Western blot analysis, frozen protoplasts were resuspended in extraction buffer (50 mM Tris-acetate, pH 7.4, 10 mM potassium-acetate, 1 mM EDTA, 5 mM DTT, 0.5 mM phenylmethyl-sulfonyl fluoride). S30 and P30 fractions were generated by centrifugation at 30,000 g for 30 min in a Sorvall SS34 rotor at 4 °C in a Beckman Avanti J-25 centrifuge. After addition of sample buffer (10% glycerol, 5% ß-mercaptoethanol, 2% SDS, 0.01% bromophenol blue, 75 mM Tris-HCl, pH 6.8) samples were boiled for 3 min at 100 °C. As a control, a membrane fraction was isolated out of cowpea plants inoculated with wild-type CPMV (Zabel et al., 1982). Proteins were separated on 12.5% SDS-PAGE gels. After electrophoresis, proteins were blotted onto nitrocellulose paper (Schleicher and Schull) and immunostained with antiserum raised against the 58K/MP as described by Van Bokhoven et al. (1990). As a second antiserum, we used goat-anti rabbit coupled to alkaline phosphatase (Life Technologies) and developed the blots using nitro blue tetrazolium (Life Technologies) and 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies) as substrates.

Immunofluorescence

Immunofluorescence analysis was performed as described by Sijen et al. (1995). Protoplasts were stained with anti-CPMV (Van Lent et al., 1991) or anti-58K/MP (Wellink et al., 1989). Stained protoplasts were viewed by a Nikon Optiphot 2 microscope, using UV1a (excitation filter 365/10) and B2A filter sets (excitation filter 450-490 nm).

RNA isolation and RT-PCR analysis

Total RNA was isolated out of inoculated and higher leaves of cowpea plants using "TriZol Reagent" according to the manufacturers instructions (GIBO/BRL). Per sample 1 ml of TriZol reagent was used. RNA pellets were resuspended in 25 μl H_2O. Complementary DNA (cDNA) was obtained by RT-PCR performed on total mRNA extracted from inoculated and higher leaves of cowpea plants. First strand cDNA was synthesised using an oligo(dT)15 primer. The cDNA was amplified using primers M512+ (5' ATGGAAAGCATTATGAGCCG 3'), and M1641- (5' GCCTTGGAC-AACAAAACTCG 3'). The PCR reaction contained 250 μM of each dNTP, 1 unit Taq polymerase (Boehringer), 2 μl cDNA reaction mixture and Boehringer Taq polymerase buffer. The PCR was for 32 cycles of 94 °C (1 min), 55 °C (1 min) and 72 °C (1.5 min) on a Perkin Elmer DNA Thermal Cycler. The amplified cDNA was checked for the presence of the introduced nucleotide changes by restriction enzyme analysis.

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STUDIES ON THE C-TERMINUS OF THE COWPEA MOSAIC VIRUS MOVEMENT PROTEIN

Abstract

Cowpea mosaic virus (CPMV) spreads from cell-to-cell as virus particles through tubular structures in modified plasmodesmata. The RNA 2-encoded movement protein (MP) is capable of forming similar tubular structures in protoplasts. Mutational analysis of the MP has revealed that the N-terminal and central regions of the MP are involved in tubule formation and that the C-terminal domain probably has a role in the interactions with virus particles. By constructing C-terminal deletion mutants and comoviral hybrid MPs, it was possible to delineate the C-terminal border of the tubule-forming domain to a small region between amino acids 292 and 298. Some of the tubular structures induced in protoplasts by a tripartite virus encoding wild-type MP and MP containing a small deletion in the C-terminal domain were not completely filled with virus. This indicates that the C-terminus of the MP is involved in the incorporation of virus particles in the tubule and that forefficient incorporation of virus particles all MP molecules in the tubue need to contain a functional C-terminus. A mutant virus coding for a MP in which the last 10 C-terminal amino acids were replaced by the green fluorescent protein (GFP) was able to induce tubule formation in protoplasts. These tubules did not contain virus particles, probably because the GFP interferes with the incorporation of virions into the tubule. These results suggest a model for the structure of the tubule in which the C-terminus of the MP is located inside the tubular structure, where it is able to interact with virus particles.

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Submitted for publication

Introduction

Cell-to-cell movement of plant viruses is a complex process involving the action of specialised viral-encoded movement proteins (MP). These MPs mediate the transport of viral genomes through plasmodesmata; small intercellular channels localised in the cell wall. Two general mechanisms for viral cell-to-cell spread have been described (reviewed in Lazarowitz and Beachy, 1999; Leisner et al., 1999, Citovsky, 1999). Viruses like tobacco mosaic virus (TMV) apparently spread from cell-to-cell as nucleoprotein complexes consisting of viral RNA and MP. The second mechanism for viral cell-to-cell movement is exemplified by cowpea mosaic virus (CPMV), which spreads from cell-to-cell as virus particles through tubular structures. These tubular structures traverse the cell wall and are localised in modified plasmodesmata (van Lent et al., 1990). Similar tubular structures containing virus particles have been found in plants infected with other como- (Kim and Fulton, 1971, Tomenius and Oxelfelt, 1982) nepo- (Walkey and Webb, 1968), caulimo- (Kitajima et al., 1969), badna- (Cheng et al., 1998), sequi- (Murant et al., 1975), tospo- (Kormelink et al., 1994) and oleaviruses (Castellano et al., 1987). Tubular structures are also formed on protoplasts infected with these viruses, as shown, among others, for the comoviruses CPMV (van Lent et al., 1991) and red clover mottle virus (RCMV) (Kasteel, 1999), the nepovirus grapevine fanleaf virus (GFLV) (Ritzenthaler et al., 1995) and the caulimovirus cauliflower mosaic virus (CaMV) (Perbal et al., 1993). For several of these viruses it has been shown that the MP is the only viral product needed for tubule formation in protoplasts (Wellink et al., 1993; Perbal et al., 1993; Grieco et al., 1999). Furthermore, tubular structures are also induced in insect cells transiently expressing viral MPs, suggesting that no plant specific proteins are involved in the process of tubule formation (Storms et al., 1995; Kasteel et al., 1996).

The CPMV genome consists of two single-stranded, positive-sense RNA molecules of 5889 nt (RNA1) and 3481 nt (RNA2), which are separately encapsidated in isometric particles with a diameter of 28 nm. Both RNA molecules are translated into large polyproteins, which are cleaved by an RNA1-encoded proteinase. The proteins encoded by RNA1 have a function in viral replication. RNA2 is translated into two polyproteins of 105K and 95K, which are cleaved into four mature products: the 58K protein involved in replication of RNA2, the 48K MP and the L and S capsid proteins (for a review about the molecular biology of CPMV see Goldbach and Wellink, 1996). Mutational analysis of the MP has indicated the presence of several functional domains (Lekkerkerker et al., 1996; Bertens et al., 2000). The N-terminal and central regions of the MP are involved in tubule formation. Within this domain, regions have been

identified that are involved in cell membrane targeting (around residues 92-93 and 121-162) (Bertens et al., 2000). Deletion analysis showed that this domain ends between residues 285 and 313 (Lekkerkerker et al., 1996). A mutant MP lacking amino acids 313-331 is still able to form tubular structures in protoplasts. However, these tubules do not contain virus particles, suggesting that the C-terminus of the MP is involved in an interaction between MP and virus particles (Lekkerkerker et al., 1996). The C-terminus also has a role in cleavage of the MP from the polyprotein precursor (Gopinath et al., 2000).

Until now, information about the structural organisation of the tubular structures induced by the CPMV and other viral MPs is limited. No MP has been crystallised and structural information has been derived from structural predictions, sequence comparisons and by mutational analysis. Although MPs of como-, nepo-, caulimo-, and tospoviruses all can form tubular structures in protoplasts, they do not share obvious sequence homology. Thomas and Maule (1995) proposed, based on computational analysis, that como- and caulimoviral MPs have a structural similarity. For both viruses it has been suggested that the C-terminus of the MP is exposed on the inner face of the tubule.

In this study we determined the C-terminal border of the tubule-forming domain more precisely and studied the effects of exchanging this domain between the CPMV and red clover mottle virus (RCMV) MP on tubule formation and viral replication. Furthermore, we present data confirming that the C-terminus of the CPMV MP interacts with virus particles and is located inside the tubular structure.

Results

Demarcation of the tubule-forming domain

In previous experiments it has been determined that the C-terminus of the tubule forming domain of the CPMV MP is located between amino acid residues 285 and 313 (Lekkerkerker et al., 1996). To delineate this border more precisely, we generated two deletion mutants lacking 44 (M48ΔC5) and 51 (M48ΔC6) amino acids from the C-terminus of the MP (Figures 1A and 1B). Protoplasts were inoculated with transcripts of pTB1G, encoding wild-type RNA1, and pTM48ΔC5 or pTM48ΔC6, or with transcripts of pTM1G (encoding wild-type RNA2) as a control (in the remainder of the manuscript we will only mention the source of RNA2). Protoplasts were analysed by immunofluorescence microscopy 42 hours post inoculation (h.p.i.) using an antiserum raised against the 58K protein expressed in *E. coli* (Kasteel et al., 1996). The proportion

of protoplasts inoculated with the two deletion mutants and wild-type RNA2 respectively, which stained with anti-MP serum was similar, suggesting that the deletions did not affect RNA2 replication (Table 1). Tubular structures were detected in protoplasts inoculated with wild-type RNA2 (Figure 2A) or with M48ΔC5 (data not shown). No tubular structures were detectable in protoplasts inoculated with M48ΔC6, which codes for a MP that is 7 amino acids shorter in comparison with the MP coded by M48ΔC5. Instead, in most protoplasts the cell membrane was strongly labelled. In a subset of the protoplasts fluorescent spots were detectable on the cell membrane (Figure 2C), and occasionally short tubular structures (Figure 2B). From the analysis of these two mutants, we concluded that the C-terminus of the tubule-forming domain of the CPMV MP is located between amino acids 292 and 298. Remarkably, whereas the nucleus was fluorescent in protoplasts inoculated with wild-type RNA2, this was not the case in protoplasts infected with either of the two deletion mutants, and also not in protoplasts inoculated with M48ΔC3 that codes for a MP lacking 59 amino acids of the C-terminus (Wellink unpublished).

Table 1: Immunofluorescence analysis of protoplasts inoculated with CPMV MP deletion mutants and CPMV-RCMV MP hybrids

Protoplasts inoculated with RNA1 and	Fluorescent cells (%) with			Tubular structures[1]
	α-110K serum	α-48K serum	α-CPMV serum	
RNA2	60	30	35	+
M48ΔC5	60	30	ND[2]	+
M48ΔC6	60	30	ND	-
MCRH-1	60	2	2	-
MCRH-2	60	1	3	+

[1] as determined by immunofluorescence
[2] ND: not determined

Construction and analysis of hybrid MPs

Alignment of the CPMV and RCMV MP sequences revealed that 39% of the amino acids of the two proteins are identical, while another 27% are functionally homologous (Bertens et al., 2000; data not shown). The regions with the highest homology are found in the N-terminal and central parts of the MPs, which have been shown for the CPMV MP to be involved in tubule formation. The two C-termini are less conserved. To test whether the tubule-forming domain is conserved between different comoviral MPs, the tubule-forming domain of the CPMV MP was exchanged for the corresponding region of

the MP of RCMV. RCMV forms, like CPMV, tubular structures in infected plants (Tomenius and Oxelfelt, 1982; Shanks et al., 1989) and protoplasts (Kasteel, 1999).
MCRH-1 contained RCMV MP sequences coding for amino acids 1-293 (corresponding to CPMV MP amino acids 1-291) and CPMV MP sequences coding for amino acids 294-342, separated by two alanine residues (Figure 1B). In a second hybrid, MCRH-2, the region derived from the RCMV MP was slightly larger. MCRH-2 encodes for a MP that consists of RCMV MP residues 1-299 (analogous to CPMV MP residues 1-297) and CPMV MP residues 298-342 (Figure 1). Protoplasts were inoculated with MCRH-1 or MCHR-2 and analysed by immunofluorescence 42 h.p.i.. In these experiments, a polyclonal serum recognising the C-terminal 30 residues of the overlapping 58K/MP proteins was used (Wellink et al., 1987). In cells inoculated with MCRH-1, fluorescence was uniformly distributed through the protoplasts (Figure 2D).
In a subset of the protoplasts the periphery was also labelled, and occasionally fluorescent spots similar to those observed for M48ΔC6 were found, but tubular structures were not detected, suggesting that MCRH-1 was not able to induce the growth of tubular structures in protoplasts. In protoplasts inoculated with MCHR-2 fluorescent tubular structures were detected (Figure 2E). This shows that the tubule-forming domain of the two comoviral MPs can act autonomously and that the tubule-forming domain of the RCMV MP ends between amino acids 293-299 (corresponding to CPMV MP residues 291-297).
Remarkably, in protoplasts infected with either MCRH-1 or MCRH-2, the nucleus was not labelled with antisera against the MP. Furthermore, the percentage of protoplasts that were labelled with anti-MP and anti-CPMV was rather low compared to protoplasts inoculated with transcripts of wild-type RNA2 (Table 1), suggesting that the introduction of RCMV sequences into CPMV RNA2 has an effect on viral replication. This was confirmed by Western blot analysis of extracts of protoplasts inoculated with MCRH-1 and MCRH-2 on which the CPs were barely detectable (data not shown). Although MCRH-2 was able to induce the formation of tubular structures in protoplasts, MCRH-2 was not able to infect cowpea plants. Plants remained symptomless, and no specific products could be recovered from inoculated leaves by reverse-transcriptase polymerase chain reaction (RT-PCR) analysis using primers specific for the RCMV sequence present in MCRH-2 (data not shown). Attempts to determine whether the tubular structures made by MCRH-2 contained virus-like particles using electron microscopy were not successful, due to the low number of infected protoplasts present in the samples.

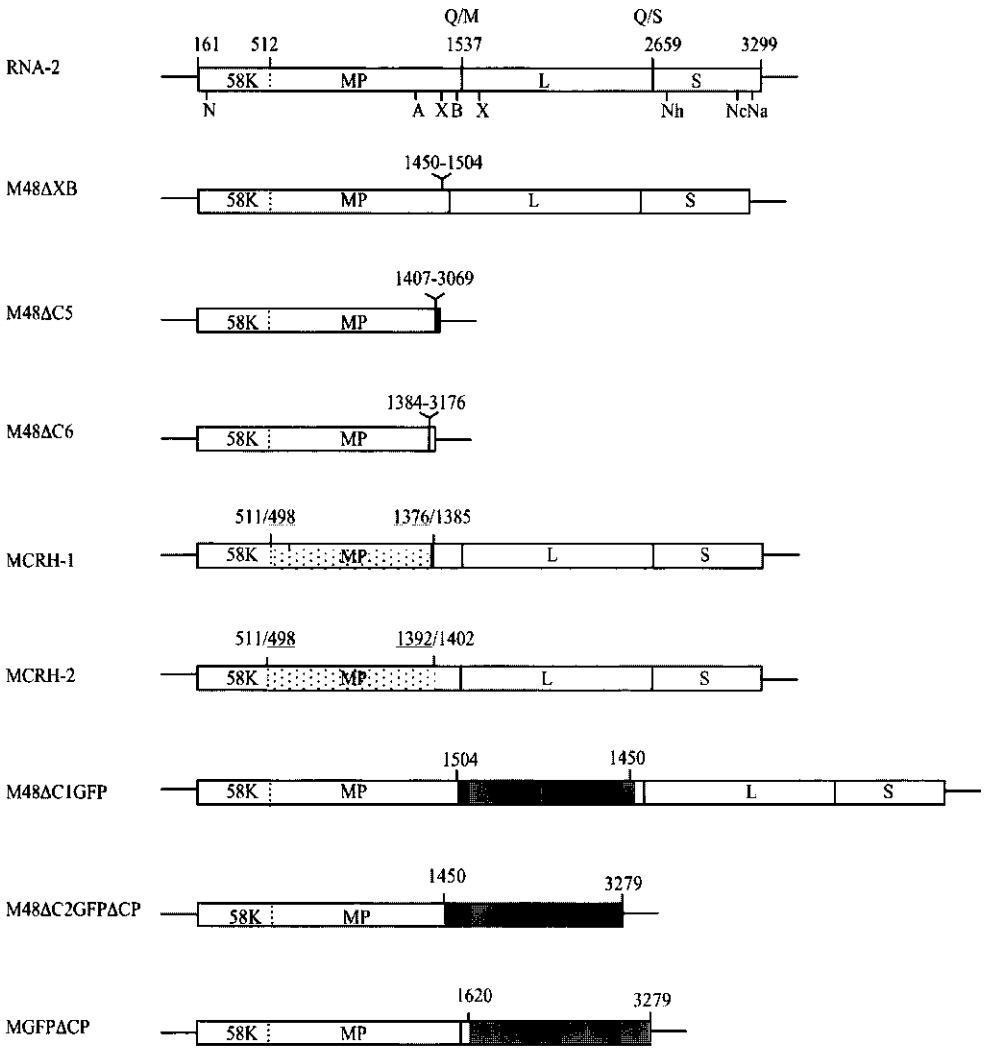

Figure 1: (A) Schematic representation of wild-type CPMV RNA2 and the mutants analysed in this study. Bars represent RNA-2 encoded open reading frames. Numbers above the bars are CPMV residues, except when underlined (RCMV residues). Black bars represent regions containing an alternative reading frame of the S protein and stippled areas represent RCMV sequences. The positions of restriction enzymes used for cloning purposes are indicated below the RNA-2 bar. Abbreviations: A: *Afl*II, B: *Bam*HI, N: *Nar*I, Nc: *Nco*I, Na: *Nae*I, Nh: *Nhe*I and X: *Xho*I. (B; next page) Overview of the C-terminal amino acid sequences of the tubule-forming domain of the MPs coded by CPMV, RCMV, M48ΔC5, M48ΔC6 and the two hybrid viruses. Residues derived from an alternative open reading frame in the S CP are shown in *italic* (in lower case), residues derived from the S CP are in lower case, RCMV residues are underlined and two alanine residues introduced into MCRH-1 during cloning are indicated in bold. Amino acid residues conserved between the CPMV and RCMV MPs are indicated above the sequence and chemically similar residues are indicated with #. ± indicates that in a subset of protoplasts spots are formed at its periphery.

Figure 1B

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consensus      .##S.#..#.K#.SL#Y#I#G..#.N#.#...#.#.E..#...        tubules
M48C5        LSNSCNAHFVKGESLKYSISG fmgqsdhlvs                       +
M48C6        LSNSCNAHFVKGESgnilmppfplstetppllkfrfrdiersk           ±
MAM7          LSNSCNAHFVKGESAAYSISGKEAENHAVHATVVSREGASAAP           +
CPMV     278 LSNSCNAHFVKGESLKYSISGKEAENHAVHATVVSREGASAAP 320      +
RCMV     280 KTGSLSSILSKSKSLRYTIGGSKPKNK.LADKAHNEEAETSDS 322      +
MCRH-1       KTGSLSSILSKSKSAAYSISGKEAENHAVHATVVSREGASAAP           ±
MCRH-2       KTGSLSSILSKSKSLRYTIGGKEAENHAVHATVVSREGASAAP           +
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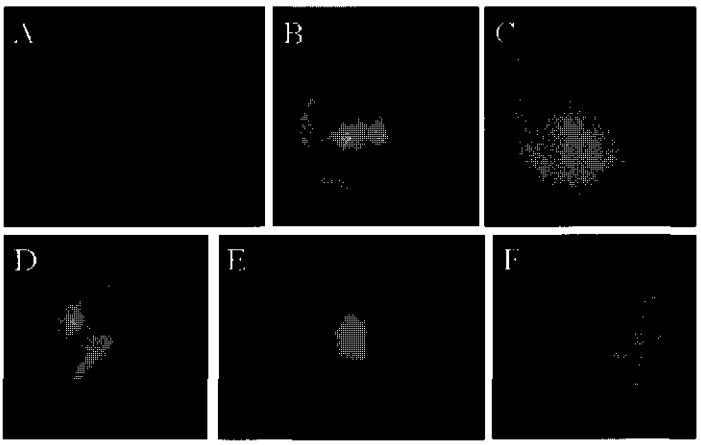

Figure 2: Immunofluorescent analysis of protoplasts inoculated with transcripts of CPMV RNA1 and wild-type RNA2 (A), M48ΔC6 (B, C), MCRH-1 (D) or MCRH-2 (E) and a confocal laser scanning microscopy of living protoplasts inoculated with CPMV RNA1 and M48ΔC1fGFP (F) 42 h.p.i.. Fourty-two h.p.i. protoplasts were labelled with anti-MP serum raised against a peptide coding for the 30 C-terminal residues of the MP (A, B, C) or the RNA-2 encoded 58K protein expressed in *E. coli* (D, E). GAR-FITC was used as a secondary antibody.

Studies on the role and localisation of the C-terminus of the CPMV MP

Lekkerkerker et al. (1996) suggested that the C-terminus of the CPMV MP might be involved in the interaction between MP and virus particles. This idea was based on the analysis of mutant M48ΔXB, which codes for a MP lacking amino acids 314-331. Upon infection of protoplasts with this mutant RNA2, tubular structures devoid of virus particles are formed and M48ΔXB is not able to infect plants. The effect of the mutation of the MP was further examined by testing whether it was possible to complement the mutation through the addition of wild-type MP. For this, plants were inoculated with a mixture of RNA1, M48ΔXB and MGFPΔCP (Figure 1A). In this tripartite virus, the CPs

and the mutant MP are supplied by M48ΔXB, and wild-type MP and a green fluorescent protein (GFP) marker by MGFPΔCP. Since both the wild-type and mutant MPs induce tubular structures in protoplasts, we anticipated that in a mixed infection they will form hybrid tubules in plants. Using a handhold UV lamp, green fluorescent spots could be detected on plants inoculated with transcripts of these mutants from 4 days post inoculation (d.p.i.) onwards. The sizes of the fluorescent spots were similar to those formed on plants infected with CPMV-TRI (data not shown; Verver et al., 1998). Western blot analysis of protoplasts inoculated with extracts of infected plants indicated the presence of wildtype and mutant MPs (MP and MPΔXB in Figure 4, lane 2). The ratio between truncated and wild-type MP was not 1:1 in these samples, suggesting that the MP coded by M48ΔXB is less stable or is produced in lower amounts. These results show that the defect of the MP encoded by MΔXB can efficiently be complemented by wild-type MP. Subsequently, the tubular structures induced by this tripartite virus in cowpea protoplasts were examined using electron microscopy. While the virus particles in tubules formed by wild-type MP are always nicely arranged (Figure 3A), and the tubules formed in protoplasts inoculated with MΔXB alone do not contain virus particles (Lekkerkerker et al., 1995), a proportion of the tubules formed in protoplasts inoculated with the tripartite virus were not completely filled (arrows in Figure 3B). Other tubular structures in these protoplasts were indistinguishable from wild-type (data not shown). These results indicate that hybrid tubules are formed in protoplasts upon infection with MΔXB and MGFPΔCP and that the C-terminus of the CPMV MP is involved in the incorporation of virus particles in the tubular structures.

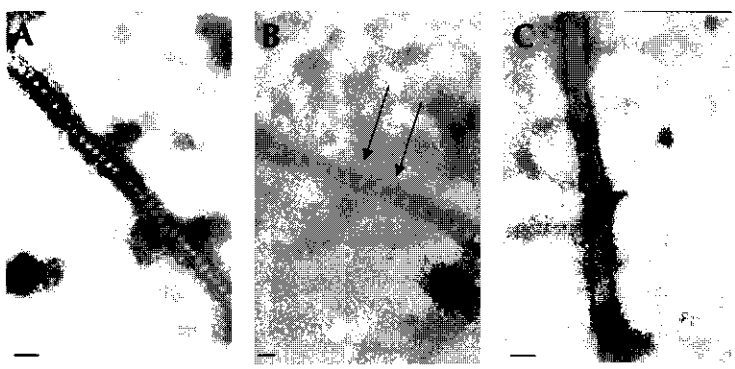

Figure 3: Electron micrographs of tubular structures formed in protoplasts inoculated with wild-type RNA2 (A), MΔXB + MGFPΔCP (B) and M48ΔC1fGFP (C). Arrows in B indicate irregularly filled segments on a tubular structure. Bars in (A) and (B) represent 60 nm, in (C) 30 nm.

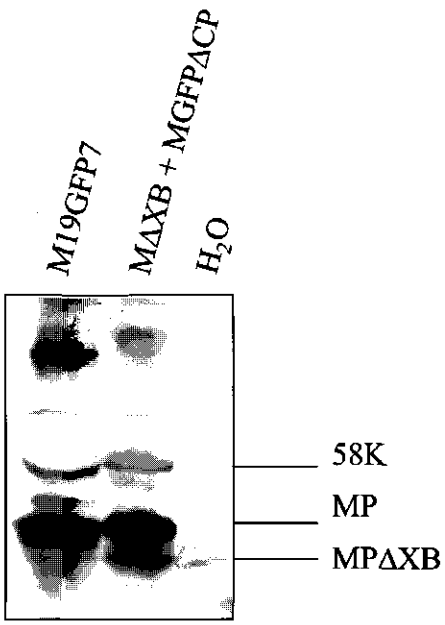

Figure 4: Western blot analysis of total extracts of cowpea protoplasts isolated 42 h.p.i. inoculated with M19GFP7 (lane 1), M48ΔXB and MGFPΔCP (lane 2) or H_2O control (lane 3). The blot was probed with an antiserum raised against the 58K protein, alkaline phosphatase-conjugated secondary antibody and developed with BCIP-NBT reagent. The positions of the 58K, MP and MPΔXB are indicated on the right.

More insight in the organisation of the tubular structure and the localisation of the C-terminus of the MP in the tubule was obtained by analysing the properties of two MP:GFP fusion products. In pTM48ΔC1fGFP, the coding region of GFP replaced the RNA2 residues coding for amino acids 332-342 of the MP. An artifical QM cleavage site was engineered to allow correct processing between MP:GFP and L CP (Figure 1). Cowpea protoplasts were inoculated with transcripts of pTM48ΔC1GFP. Green fluorescence became detectable from 24 h.p.i. onwards, as monitored by CLSM. At both 24 and 42 h.p.i., fluorescent tubular structures extended from the surface of inoculated protoplasts (Figure 2F). Tubular structures were also detected in protoplasts inoculated with transcripts of a second MP-GFP fusion construct, pTM48ΔC2fGFPΔCP (Figure 1A), coding for a MP in which the 29 C-terminal amino acids had been replaced by GFP (data not shown). So, the presence of GFP approximately 21 amino acids from the C-terminus of the tubule-forming domain of the MP has no effects on the ability of

this domain to form tubular structures. To investigate the potential of an MP:GFP fusion protein to facilitate cell-to-cell movement of CPMV, extracts of protoplasts inoculated with M48ΔC1fGFP were inoculated onto cowpea plants. Infections remained restricted to single-cells (data not shown), suggesting that the fusion of GFP to the MP blocked cell-to-cell movement. Examination of tubular structures induced on protoplasts by M48ΔC1fGFP using electron microscopy showed that none of the investigated tubules contained virus-like particles (Figure 3F). Apparently, the fusion of GFP to the MP prevents the incorporation of virions in the tubules.

Discussion

Previous studies have indicated that the N-terminal and central regions of the CPMV MP are involved in tubule-formation, while the C-terminus probably interacts with virus particles (Lekkerkerker et al., 1996; Bertens et al., 2000). In this study, analysis of two new deletion mutants and hybrid CPMV/RCMV constructs showed that the C-terminus of the tubule-forming domain of the CPMV MP is located between amino acids 292 and 298 (Figure 1B). Deletion mutant M48ΔC5, which codes for a MP lacking amino acids 298-342, was able to form tubular structures in protoplasts reminiscent to those formed by wild-type MP. In contrast, no such tubular structures were found in protoplasts inoculated with M48ΔC6, a mutant coding for a MP lacking amino acids 292-342. This MP was targeted to the cell membrane where it occasionally accumulated in fluorescent spots, which might be regarded as an attempt to form tubules. Indeed, in a minority of protoplasts, short tubular structures were detected. Apparently, the region around amino acid 292 has a role in the assembly of the tubular structures. Tubule formation was not affected in protoplasts inoculated with mutant MAM7, in which amino acid residues 292 and 293 have been replaced by alanine residues (Bertens et al., 2000), while a mutant lacking amino acids 285-342 (M48ΔC3) did not form tubular structures at all (Lekkerkerker et al., 1996). The tubule-forming domain is conserved between the CPMV and RCMV MPs, since MCRH-2, which contained RCMV MP residues 1-299 (analogous to CPMV residues 1-297) can form tubular structures. In addition, a RCMV:CPMV hybrid MP containing a tubule-forming domain with a small deletion (MCRH-1), had a similar phenotype in protoplasts as M48ΔC6. The MPs of both mutants accumulated in spots in the cell membrane, but were not able to form long tubular structures, suggesting that formation of these spots precedes tubule formation.

It has been suggested that the C-termini of the CPMV and GFLV MPs specifically interact with virus particles, enabling incorporation of virions in the tubular structure (Lekkerkerker et al., 1996; Belin et al., 1999). Tubules induced by mutant M48ΔXB in protoplasts do not contain virus particles (Lekkerkerker et al., 1996). This has been attributed to a deletion in the domain of the MP interacting with virus capsids. A tripartite virus, consisting of RNA1, MGFPΔCP (which codes for wild-type MP) and M48ΔXB, was quite efficient in cell-to-cell spread, although a subset of the tubular structures induced by this virus is irregularly filled with virus particles. Western blot analysis suggested that there is a higher amount of wild-type MP present in the tubular structures. This suggests that the minority of mutant MP (lacking a functional C-terminus) is responsible for the observed phenotype, i.e. the irregularly filled tubules. This indicates that for an effective incorporation all MP molecules present in a tubular structure need to contain a functional C-terminus.

Fusion of GFP to amino acid 332 (M48ΔC1fGFP) or 312 (M48ΔC2fGFPΔCP) of the MP did not abolish tubule formation in protoplasts. Likewise, GFP has been fused to the C-terminus of the MPs of alfalfa mosaic virus (AMV) (Zheng et al., 1997), olive latent virus-2 (OLV-2) (Grieco et al., 1999) and cucumber mosaic virus (CMV) (Canto and Palukaitis, 1999). In all these cases this did not affect the subcellular targeting and tubule formation, suggesting that the C-termini of these MPs are not directly involved in tubule formation and that these MPs have a similar domain structure. In contrast to these results, fusion of MP to the C-terminus of the CaMV MP prevented the formation of tubular structures in insect cells (Thomas and Maule, 1999b). This can be overcome by addition of unfused MP into the same cell. The observation that M48ΔC1fGFP was unable to infect plants can be explained by the finding that the tubular structures formed in protoplasts did not contain virus particles. The "empty" tubules of M48ΔC1fGFP are probably the result of steric hindrance by GFP located at the inside of the tubular structure, preventing an essential interaction between MP and CP. This is in accordance with the result of Thomas and Maule (1995, 1999a) for CaMV, who showed that the C-terminus of the CaMV MP is not necessary for tubule formation and suggested that the C-terminus of the MP is located inside the tubule.

Since replication and translation of the CPMV RNAs are tightly linked, Western blotting was used to assess the replication of (mutant) RNA2 molecules (Wellink et al., 1994). MCRH-1 and MCRH-2 only replicated at a low level when compared to wild-type RNA2. Apparently, the introduction of RCMV sequences into CPMV RNA-2 negatively influences replication. This was somewhat unexpected, since CPMV RNA-2 molecules carrying large deletions in the MP coding region are still able to replicate normally (Wellink et al., 1989) and the CPMV and RCMV RNA2 molecules are closely related (Haudenshield and Palukaitis, 1998). Probably, this effect is caused by the fusion of the

RCMV residues to the 10K-specific portion of the 58K protein. In this respect it is worthwhile to mention that the replication of a RNA2 molecule encoding a fusion between the N-terminal 10K of the 58K protein and GFP was also severely affected in protoplasts (J. Verver and J. Wellink, unpublished results). Furthermore, it is remarkable that in protoplasts inoculated with M48ΔC3, M48ΔC5, M48ΔC6, MCRH-1 or MCHR-2, the mutant 58K is not targeted to the nucleus. This suggests that a nuclear localisation of the 58K protein is not a prerequisite for viral replication and that the reduced levels of replication observed for the MCRH-1 are not due to the altered localisation of the 58K protein. Furthermore, these results suggest that, while the 10K region is essential for replication, regions outside the 10K region of the 58K protein are involved in targeting of this protein to the nucleus.

Materials and methods

Construction of mutant viruses

Routine manipulation of DNA was done according to Sambrook et al. (1989). All enzymes used were purchased from Life Technologies (Eggenstein, Germany) or Boehringer Mannheim (Mannheim, Germany). Plasmid pTB1G, containing a full-length cDNA copy of CPMV RNA1 has been described by Eggen et al. (1989). All mutations, except where indicated, were introduced in plasmid pTM1G that contains a full-length cDNA copy of CPMV RNA-2 (Eggen et al., 1989). For construction of pTM48ΔC5, we have made use of pTMP3. This mutant was created by site-directed mutagenesis according to the method of Kunkel (1985) using the oligonucleotide 5'-GAAATACTCTATTCGAACAAAGC-3' resulting in an unique *Nsp*V restriction site (underlined in the primer) at position 1409 of RNA2 in pTM1G. pTMP3 was digested with *Nsp*V and *Nco*I (nt 3068) and religated to create pTM48ΔC5, in which residues 1409–3069 were deleted. This resulted in a deletion of 44 residues in the C-terminus of the MP, which became fused to 10 amino acids derived from an alternative reading frame in the S gene. For construction of mutant pTM48ΔC6, pTMAM7 (Bertens et al., 2000) was digested with *Eco*47III (introduced at nt 1385 in pTMAM7) and *Nae*I (nt 3172) and religated. In pTM48ΔC6, the fragment between nucleotides 1389 and 3175 was deleted, which resulted in a deletion of 48 amino acids in the C-terminus of the MP, which became fused to 41 C-terminal residues from the S protein (see figure 1).
To construct pTMCRH-1, a 1.1-kb *Nar*I (nt 214)-*Afl*II (nt 1301) fragment of pTMΔAUG2 (Wellink et al., 1993) was ligated into the 4.7 kb *Afl*II-*Nar*I fragment of pTAM8 resulting

in pTMΔAUG2AM7. The unique *Eco*RI (nt 512) and *Eco*47III (nt 1385) restriction sites of this mutant were used to excise the CPMV MP coding sequence and to insert the homologous part of the RCMV MP coding sequence. The RCMV sequence was amplified by polymerase chain reaction (PCR) from plasmid pBS21 (kindly provided by Dr. G.P. Lomonossoff) using primers R498+ (5'-GTGAATTCATGGCACAAG-AAATTTTGAAAC-3') and R1376- (5'-TTTAGTACTTTTACTCTTACTCAGTATAG-3') and digested with *Eco*RI and *Sca*I (unique sites underlined in the primers). pTMCRH-1 codes for a MP containing RCMV residues 1-293 and CPMV residues 294-342, separated by two alanine residues.

For construction of pTMCRH-2, the RCMV MP coding sequence was amplified by PCR using primers R498+ and R1392- (5'-ATATATCGATCGTGTACCTCAAACTTT-3') and cloned into a pGEM-T vector to create pGEM-TR2. A CPMV RNA-2 cDNA fragment, which was obtained by PCR using primers M1393+ (5'-ATATATCGATCGGAGGC-AAAGAAGC-3') and M3450- (5'-GAAGGGACGACCTG-CTAAAC-3') was also ligated into pGEM-T generating pGEM-TC2. The inserts in the two pGEM-T constructs were isolated after digestion with *Pvu*I (underlined in primer R1392-) and *Eco*RI (pGEM-TR2) and *Pvu*I (underlined in primer M1393+) and *Nhe*I (pGEM-TC2) and cloned into pMΔAUG2 digested with *Eco*RI (nt 512) and *Nhe*I (nt 2708) to create pMCRH-2. The MP encoded by MCRH-2 contained RCMV residues 1-299 and CPMV residues 298-342.

For construction of pTM48ΔC1GFP, pTM1G and pTMGFPΔCP1Bg (Verver et al., 1998) were digested with *Bam*HI (nt 1504) and *Xho*I (nt 1620), respectively. Both linearised plasmids were treated with Mung Bean Nuclease for 30 seconds at 37 °C, purified using Qiagen spin columns (Qiagen, Studio City, CA) and digested with *Mlu*I (present in the vectors). The small 1.55 kb fragment obtained from pTM1G was ligated to the large 3.2 kb fragment obtained from pTMGFPΔCP1Bg resulting in pTMGFPΔCP1BgΔBX. Next, the 4.3 kb *Afl*II/*Bam*HI fragment of pTM1G was ligated to the 2.2 kb *Bgl*II/*Afl*II fragment of pTMGFPΔCP1BgΔBX to obtain pTM48ΔC1fGFP (Figure 1).

pTMGFPΔC2fGFPΔCP was created by digestion of pTMGFPΔCP (Verver et al., 1998) with *Xho*I and religated, resulting in the deletion of nucleotides 1447-1621 and the fusion of the MP and GFP coding regions (Figure 1).

The constructions of pTMGFPΔCP (Verver et al., 1998), pTM48ΔXB (Lekkerkerker et al., 1996) and pTM19GFP7 (Gopinath et al., 2000) have been described previously.

Inoculation and analysis of protoplasts and plants

Cowpea protoplasts were isolated and inoculated with *in vitro* transcripts of the different cDNA clones as described by van Bokhoven et al. (1993). Protoplasts were inoculated with 1 μg each of transcripts derived from pTM1G (a plasmid encoding a

full-length clone of CPMV RNA-1) (Eggen et al., 1989) and wild-type or mutant RNA-2 plasmids. RNA transcripts were generated as described previously (Bertens et al., 2000). Protoplasts were cultured under continuous illumination at 25 °C. An aliquot of the protoplast sample was used for immunofluorescence analysis 42 hours post-inoculation and the remainder was harvested by centrifugation at 3,000 rpm and stored at – 80 °C for further use. Immunofluorescence was performed as described by Bertens et al. (2000). Two types of anti-MP sera were used. One serum was raised against a peptide comprising the C-terminal 30 amino acids of 58K/MP (Wellink et al., 1987) and the other was raised against a bacterially expressed 58K protein (Kasteel et al., 1996). Protoplast samples were investigated for fluorescence using a Nikon Optiphor 2 microscope (using the UV1A filter set; excitation filter, 365/10 nm) or a Zeiss LSM512 confocal microscope.

Plants were inoculated with protoplast extracts as described earlier (Bertens et al., 2000). Inoculated plants were investigated daily for symptoms and fluorescence by a hand held UV lamp. Small fluorescent spots were visualised by confocal laser scanning microscopy.

Samples for Western blot analysis were generated according to Bertens et al. (2000). Western blotting was performed as described by Gopinath et al. (2000).

Electron microscopical analysis

Copper grids (400-mesh) with a carbon-coated formvar support film were exposed to a glow discharge in air for 10 seconds and incubated in a 0.05% (w/v) poly-L-lysine solution for 5 min. Excess solution was removed with filter paper, and the grids were air-dried. Samples of inoculated protoplasts were placed on these grids and incubated for 3 min. The grids were then rinsed with double-distilled water and fixed with 2% (2/v) phosphotungstic acid (PTA) in double distilled water, pH 5.5. Excess PTA was removed with filter paper, and the grids were left to dry. The samples were examined using a Philips CM12 electron microscope.

Acknowledgements

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INTRACELLULAR DISTRIBUTION OF COWPEA MOSAIC VIRUS MOVEMENT PROTEIN:GREEN FLUORESCENT PROTEIN FUSIONS

Abstract

Plants infected with cowpea mosaic virus (CPMV) contain typical tubular structures filled with virus particles in modified plasmodesmata. These tubules mainly consist of virus-specific movement protein (MP) and are involved in cell-to-cell spread of CPMV. Similar structures are also formed in infected protoplasts. In order to study the targeting and localisation of the MP in living cells, the MP was fused to the green fluorescent protein (MP:GFP). In protoplasts, a virus coding for MP:GFP (MPfGFP) induced the formation of tubular structures, showing that subcellular targeting and tubule formation are not affected by fusion of GFP to the C-terminus of the MP. MPfGFP was not able to spread in plants, probably because the fusion of GFP to the MP interferes with MP-virion interaction. In epidermal cells of inoculated plants, MP:GFP mainly accumulated in fluorescent spots in the cell wall, which may represent short tubular structures in modified plasmodesmata. Furthermore, the CPMV MP was found to be able to traffick from cell-to-cell by itself. Infections with M8GFP7, a virus which produces both MP and MP:GFP in about 1:1 ratio, were limited to a few epidermal cells. Studies on the localisation of mutant MP:GFP and co-inoculation experiments with mutant MP:GFP and wild-type MP revealed that the N-terminal and central region of the MP are involved in targeting to the cell membrane in protoplasts and to the cell wall/plasmodesmata in plants and that the C-terminal region of the tubule-forming domain of the MP is essential for tubule initiation and elongation.

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Introduction

To establish a systemic infection, plant viruses must spread from the sites of initial infection to other, uninfected parts of the plant. This process occurs in two distinct steps: spread from the initially infected cell to neighbouring cells (short-distance or cell-to-cell movement) and, after the infection reaches the vasculature, long-distance movement to the higher leaves and/or the root system of the host plant. Plant viruses code for a class of specific proteins, called movement proteins (MPs), which in most cases are not involved in viral replication or encapsidation, but have specific roles in cell-to-cell movement. By the action of these MPs plasmodesmata are modified to enable transport of virus particles or non-virion complexes of viral RNA and proteins, from infected to neighbouring, uninfected cells (for recent reviews see Lazarowitz and Beachy, 1999, Leisner, 1998).

Cowpea mosaic virus (CPMV), the type member of the genus comovirus, has a single-stranded, positive-sense RNA genome that is divided over two separately encapsidated molecules, RNA1 (5889 nucleotides) and RNA2 (3481 nucleotides). The RNAs code for large polyproteins that are cleaved into 15 intermediate and final cleavage products by an RNA1-encoded proteinase (reviewed in Goldbach and Wellink, 1996). Both RNAs are needed for viral infectivity, with RNA1 coding for proteins involved in viral replication and RNA2 coding for proteins involved in spread of the virus through the plant (Wellink and van Kammen, 1989, Verver et al., 1998). In CPMV-infected cells, tubular structures containing virus particles have been detected in modified plasmodesmata, and immuno-electron microscopical analysis showed that the MP is a major component of these tubular structures (Van Lent et al., 1990). Similar tubular structures are also found on CPMV-infected protoplasts and tubules without particles are formed on protoplasts upon transient expression of the MP only (Van Lent et al., 1991, Wellink et al., 1993a). Mutational analysis of the CPMV MP has indicated the presence of several functional domains (Lekkerkerker et al., 1996, Bertens et al., 2000). The C-terminus of the MP is thought to interact with virus particles, whereas the remainder of the protein has a role in tubule formation. Furthermore, regions involved in cell membrane targeting of the MP are located within the tubule-forming domain.

Recently, use of the green fluorescent reporter (GFP) has provided new insights into the mechanisms of virus movement in plants (see Oparka et al., 1996). For several viruses MP:GFP fusion products have been shown to be a useful tool to study the localisation of wild-type and mutant MPs in plants (Padgett et al., 1996, Canto et al., 1997, Erhardt et al., 1999, Satoh et al., 2000). In this study, we have fused the GFP to the C-terminus of the CPMV MP within an infectious clone of RNA2 and studied its localisation in

protoplasts and plants. Although the MP:GFP fusion protein retained the capacity to form tubular structures in infected protoplasts and is targeted to punctate spots in the cell wall of virus-infected plants, the CPMV derivatives encoding this fusion protein were not able to spread efficiently from cell-to-cell. We further show that MP:GFP is able to move from cell-to-cell, a property the CPMV MP shares with other known MPs (Waigmann et al., 1994, Ding et al., 1995, Itaya et al., 1995, Huang and Zhang, 1999, Satoh et al., 2000). In addition, the intracellular localisation of several mutant MP:GFP fusion proteins was studied in protoplasts and plants. These experiments revealed that mutations in the central region of the CPMV MP affected its subcellular targeting and identified amino acid residues involved in the initiation or elongation of tubular structures in protoplasts.

Results

Construction and characterisation of MP:GFP fusions

To study the intracellular localisation of the CPMV MP during viral infection, we constructed several viruses that encode the MP as a fusion to GFP, using pTM1G which contains a full-length cDNA clone of CPMV RNA2 (Figure 1).

In initial experiments, protoplasts were inoculated with transcripts of pTMPfGFP, that codes for a MP fused at its C-terminus to GFP (MP:GFP; Figure 1) and pTB1G, which contains a full-length cDNA clone of RNA1 (In the remainder of this paper we will only mention the RNA2 derivatives used). Membrane extracts of protoplasts isolated 42 hours post inoculation (h.p.i.) were analysed by Western blotting using an antiserum raised against the CPMV MP (Figure 2). In protoplasts inoculated with MPfGFP, bands are visible with sizes expected for the MP:GFP and 58K:GFP fusion products (Figure 2, lane 4). No bands corresponding to free MP or 58K were observed, indicating that cleavage did not occur between MP and GFP. The smaller product visible on the blot did not react with antiserum against GFP and therefore probably is a truncated MP fragment. No other bands were detected upon treatment of the blot with anti-GFP (data not shown). Consequently, the fluorescence detectable in protoplasts inoculated with MPfGFP comes from GFP fused to the MP and 58K proteins and not from free GFP.

Living protoplasts inoculated with MPfGFP were investigated by confocal microscopy. Already 12 h.p.i. green fluorescence was detectable in tubular structures that extended from the surface of protoplasts into the incubation medium (Figure 3A). Inside the cells fluorescence was detectable in the nucleus and faintly dispersed in the cytoplasm (Figure 3A).

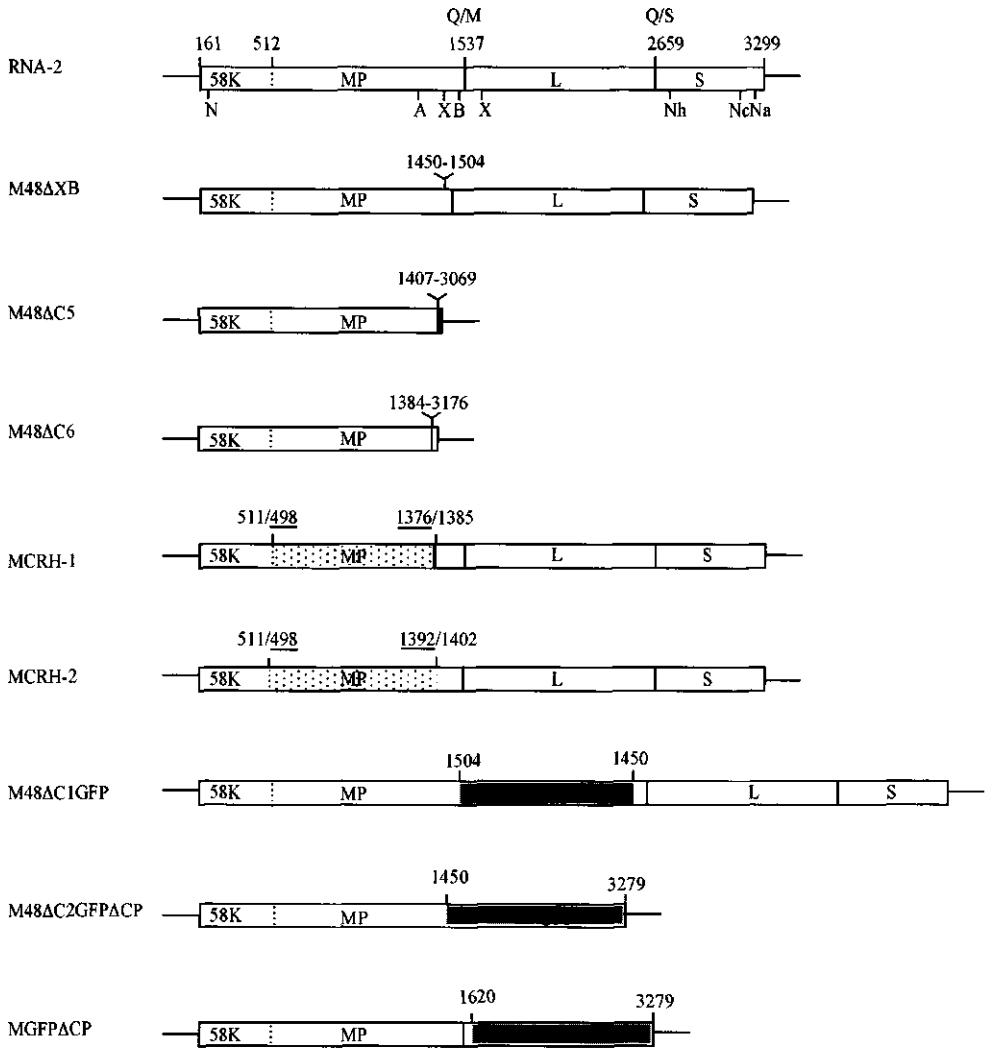

Figure 1: Overview of CPMV RNA2 constructs used in this study. Open reading frames are indicated as an open bar. Proteolytic processing sites are indicated with a black triangle, sites that are cleaved for 50% are indicated with a grey triangle. Numbers are according to wild-type RNA2. The linker sequences present between MP and GFP in MPfGFP, M8GFP7 and MPfGFPΔCP are indicated with residues derived from the L CP in normal case and residues introduced during cloning of the mutants in *italics*. The restriction enzymes used during cloning are indicated in RNA2. Enzyme abbreviations: B, *Bgl*II; Ba, *Bam*HI; N, *Nar*I; Na, *Nae*I. Other abbreviations: MP, movement protein; L, large coat protein; S, small coat protein.

In a few protoplasts, fluorescent spots were present at the periphery of the cell (data not shown). Filamentous structures or fluorescent bodies, resembling those observed in cells infected with tobacco mosaic virus (TMV) MP:GFP (Más and Beachy, 1998) were not observed. Similar patterns of fluorescence were seen 24 and 48 h.p.i. From these

results we conclude that the MP:GFP protein encoded by MPfGFP in protoplasts is correctly targeted to the periphery of the cell is incorporated into tubular structures as wild-type MP. Electron-microscopical examination of the tubular structures formed by MPfGFP revealed that the tubules did not contain virus particles (data not shown). Apparently, fusion of the MP with GFP prevents the incorporation of virus particles within the tubular structures.

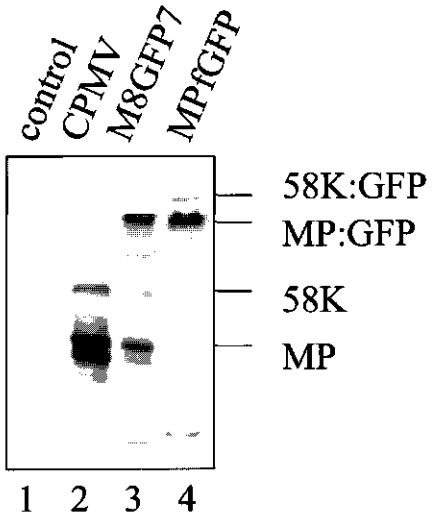

Figure 2: Immunoblot analysis of membrane extracts of protoplasts inoculated with MP:GFP mutants MPfGFP (lane 4), M8GFP7 (lane 3), CPMV (lane 2) and mock-inoculated control (lane 1) using anti-MP serum. Proteins were separated on a 12.5% SDS-PAGE gel. The positions of the MP, 58K, MP:GFP and 58K:GFP proteins are indicated.

Subcellular localisation of MP:GFP in infected plants

Next, we tested the infectivity of MPfGFP in plants. For this, 12 days old cowpea plants with fully expanded primary leaves were inoculated with MPfGFP. The intracellular localisation of the MP:GFP fusion encoded by MPfGFP was investigated by CLSM and fluorescence microscopy in plants 3 d.p.i.. At most infection sites, fluorescence seemed to be confined to single cells and was mainly present in the cell wall between two epidermal cells, where MP:GFP accumulated in prominent punctate spots (Figure 3B). These spots most likely represent accumulations of MP:GFP in plasmodesmata, where MP:GFP may induce the formation of short tubular structures. Only occasionally tubular structures protruding from the cell wall of an infected epidermal cell into the cytoplasm of a neighbouring cell were observed.

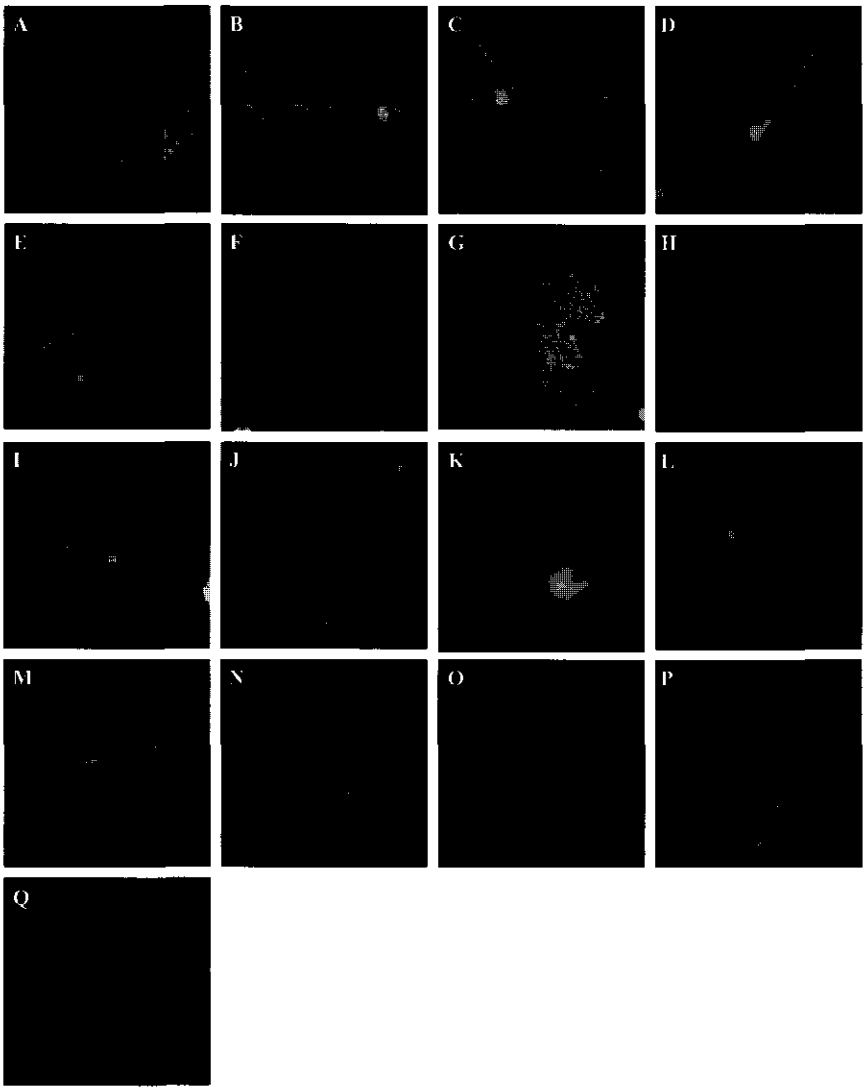

Figure 3: CLSM images of cowpea protoplasts (A, K, L, M, N) cowpea plants (B, C, D, E, G, H, I, O, P. Q) and *N. benthamiana* plants (F, J) inoculated with wildtype or mutant viruses coding form MP:GFP fusion proteins. (A) cowpea protoplasts inoculated with MPfGFP; (B) epidermal cells of cowpea plants inoculated with MPfGFP; (C) epidermal cells of cowpea plants inoculated with M19GFP0; (D) epidermal cells of cowpea plants inoculated with M8GFP7; (E) guard cells of cowpea plants inoculated with M5GFP7; (F) epidermal cells of *N. benthamiana* inoculated with M8GFP7; (G) top of epidermal cells of cowpea plants inoculated with M8GFP7; (H) epidermal cells of cowpea plants inoculated with MPfGFPΔCP; (I), detail of epidermal cells of cowpea plants inoculated with MPfGFPΔCP; (J), top of mesophyll cells of cowpea plants inoculated with M8GFP7; (K), cowpea protoplasts inoculated with

M48ΔAUG2fGFP; (L), cowpea protoplasts inoculated with MAM6fGFP; (M), cowpea protoplasts inoculated with MΔA1fGFP; (N), cowpea protoplasts inoculated with M48ΔC3fGFP; (N), epidermal cells of cowpea plants inoculated with MAM6fGFP; (O), epidermal cells of cowpea plants inoculated with MAM6fGFP; (P) epidermal cells of cowpea plants inoculated with MΔA1fGFP; (Q), epidermal cells of cowpea plants inoculated with M48ΔC3fGFP

Tubular structures were observed on the upper surface of epidermal cells (Figure 3G). In addition, fluorescence was also detected in the nucleus and faintly in the cytoplasm (Figure 3B). In addition, fluorescent punctate spots were also found in the cell wall of guard cells in leaves inoculated with MPfGFP (data not shown) or M5GFP7 (Figure 3E). M5GFP7 encodes a fusion product in which MP and GFP are separated by five residues of the L coat protein (CP). This mutant had identical biochemical properties and fluorescence patterns as MPfGFP, and therefore was not further described in this paper.

Table 1: Quantitation of GFP-expressing cells on cowpea leaves inoculated with CPMV MP:GFP fusion constructs 12 days after germination

mutant	total no. of infection sites 3 d.p.i.	no. of infected cells 3 d.p.i.			total no. of infection sites 6 d.p.i.	no. of infected cells 6 d.p.i.		
		1	2	> 2		1	2	> 2
MPfGFP	34	23 (68%)	6 (18%)	5 (15%)[1]	36	26 (72%)	6 (17%)	4 (11%)[1]
M8GFP7	48	21 (44%)	9 (19%)	18 (38%)[2]	55	15 (28%)	15 (28%)	25 (45%)[2]

[1] maximum number of cells is 3
[2] maximum number of cells is 10

Using a fluorescence microscope at most infection sites the MP:GFP fluorescence seemed to be confined to single epidermal cells at 3 days post inoculation (d.p.i.). However, at about 30% of the infection sites fluorescence was present in 2 or 3 epidermal cells (Table 1). At 6 d.p.i., the ratio between the GFP-containing single cells and clusters consisting of 2-3 cells did not differ from that observed 3 d.p.i. (Table 1). In plants infected with M19GFP7, a virus expressing GFP as a free protein, the infection spreaded rapidly and reached the systemic leaves 5-6 days post inoculation (Gopinath et al., 2000). These results indicate that the cell-to-cell spread of MPfGFP was blocked in cowpea plants. Similar results were obtained upon infection of 5 weeks old *Nicotiana benthamiana* plants with MPfGFP (data not shown). When 8 days old cowpea plants in which the primary leaves were younger and not yet fully expanded, were inoculated with MPfGFP there was slightly more extensive spread of MPfGFP: and at 7 d.p.i. approximately 50% of the infection sites consisted of 2 to 4 epidermal cells

(Table 2). Although the tubules formed in protoplasts inoculated with MPfGFP did not contain virus particles, it cannot be excluded that the limited spread of MPfGFP observed in plants is caused by sporadic incorporation of virus particles into tubular structures *in planta* followed by subsequent cell-to-cell movement of these virions. Furthermore, some of the fluorescence in neighbouring cells might be due to the trafficking of MP:GFP. Indeed, careful examination of the single cell infection sites by CLSM revealed that there is always some fluorescence, accumulating in punctate spots in the cell wall of neighbouring cells (data not shown).

To confirm the possibility that MP:GFP trafficks to adjacent cells, plants were inoculated with transcripts derived from pTMPfGFPΔCP, encoding MPfGFP but not the CPs (Figure 1). Since CPMV cannot spread from cell-to-cell in the absence of CPs, spread of the fluorescent MP:GFP signal to neighbouring cells would indicate autonomous movement of MP:GFP. Using confocal microscopy, fluorescence was found as punctate spots in the cell wall in a maximum of 2-3 neighbouring cells (Figure 3H; enlarged in Figure 3I). As a control, plants were inoculated with M19GFP0, in which GFP was fused to the N-terminus of the L CP (Figure 1). On these plants only single, strongly fluorescent epidermal cells were detected, and no fluorescence was detectable in neighbouring cells (Figure 3C). These results indicate that the CPMV MP can traffick from cell-to-cell by itself.

Table 2: Quantitation of GFP-expressing cells in epidermal cells of cowpea plants inoculated with CPMV MP:GFP constructs 8 and 12 days after germination

mutant	total no. of infection sites	no. of infected cells on leaves inoculated 8 days after germination (3 d.p.i.)			total no. of infection sites	no. of infected cells on leaves inoculated 12 days after germination (3 d.p.i.)		
		1	2	> 2		1	2	> 2
MPfGFP	98	51 (52%)	16 (16%)	31 (32%)[1]	100	72 (72%)	17 (17%)	11 (11%)[3]
M8GFP7	100	24 (24%)	24 (24%)	68 (68%)[2]	88	28 (32%)	15 (17%)	45 (51%)[4]

[1] maximum number of cells is 4
[2] maximum number of cells is 15; some mesophyll cells infected
[3] maximum number of cells is 3
[4] maximum number of cells is 10; no mesophyll cells infected

Complementation of MP:GFP by non-fused MP

To complement the defect in cell-to-cell movement of MPfGFP two approaches were taken. First, cowpea plants were co-inoculated with transcripts of pTMPfGFP and pTM19ΔCP, which codes only for the 58K and MP proteins (Figure 1). From 2 d.p.i. onwards, tiny fluorescent spots, spanning 5-10 epidermal cells, were visible on inoculated leaves (data not shown). These spots did not increase in size in time,

suggesting that the complementation was not very efficient. Mesophyll cells were not infected.

In addition, M8GFP7, in which the MP and GFP coding regions were separated by a sequence coding for 8 residues of the L CP (Gopinath et al., 2000; Figure 1) was investigated. Like MPfGFP, M8GFP7 produced fluorescent tubular structures on the surface of inoculated protoplasts (data not shown). Western blot analysis of extracts of these protoplasts indicated that approximately 50% of the MP:GFP fusion encoded by M8GFP7 was cleaved into MP and GFP (Figure 2, lane 3). Electron-microscopical analysis of the tubular structures induced by M8GFP7 suggested the presence of two different tubule populations, as approximately 40% of the tubular structures contained viral particles (Figure 4A), while the other 60% of the tubules did not (Figure 4B). In general, the tubules containing virions were irregularly formed (Figure 4A). To investigate whether the tubules induced by M8GFP7 are composed of two MPs (MP and MP8GFP) or whether there are two classes of tubular structures, each composed of a single type of MP, protoplasts inoculated with M8GFP7 were immunostained with anti-MP. As a secondary antibody, GAR-TRITC was used, which would make it possible to distinguish between tubules that only contained MP (red) and tubules that contained both MP and MP:GFP (red and green) (Figure 4). All tubular structures were found to be composed of both MP and GFP, suggesting that MP and MP:GFP were randomly incorporated into these structures (Figure 4E).

Although M8GFP7 showed more extensive spread than MPfGFP in plants, infection sites of M8GFP7 also remained restricted to only a few cells. Three d.p.i. approximately 56% of the sites consisted out of more than 1 cell, a percentage that increased slightly to 68% at 6 d.p.i. (Table 1). The largest infection sites consisted of 10-12 cells (Gopinath et al., 2000). The MP:GFP coded by M8GFP7 accumulated in fluorescent spots in the cell wall (Figure 3D), similar to those observed for MPfGFP. With M8GFP7 more fluorescence was detectable in the cytoplasm compared to MPfGFP, due to the presence of free GFP (Figure 3D). In *N. benthamiana* and in young cowpea leaves infected with M8GFP7, also some mesophyll cells became infected and occasionally long tubular structures were seen between two epidermal cells (Figure 3F) and between epidermal and mesophyll cells (Figure 3J).

Localisation of mutant MP:GFP fusions in protoplasts and plants

Several of the CPMV MP mutations described by Wellink et al., (1993b), Lekkerkerker et al. (1995) and Bertens et al. (2000) with disturbed tubule formation were introduced into pTMPfGFP, and the cellular location of the mutant MP:GFPs was investigated in protoplasts and plants.

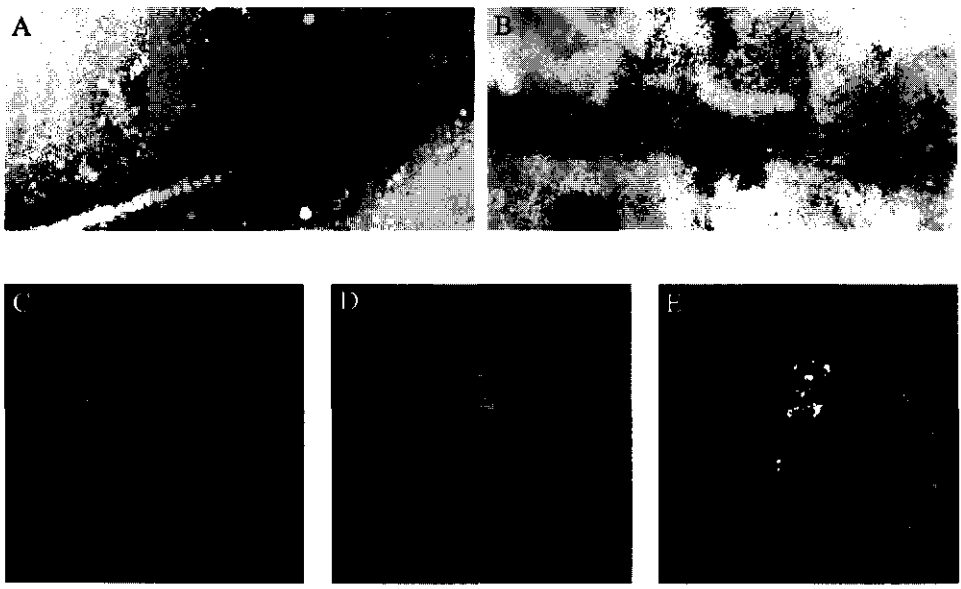

Figure 4: Analysis of tubular structures induced by M8GFP7 on cowpea protoplasts. Electromicrographs of tubular structures (A, B). CLSM images of infected protoplasts, stained with anti-MP serum (C to E). (C) green channel showing GFP fluorescence, (D) red channel showing TRITC anti-MP labelling (E) superposition of (C) and (D)(yellow).

Fluorescence in protoplasts inoculated with transcripts of pTM48ΔAUG2fGFP, which codes for a MP lacking the 4 N-terminal amino acids (see Wellink et al., 1993b), was mainly localised in the nucleus (Figure 3K). In addition, faint fluorescence was detected in the cytoplasm. Similar patterns of fluorescence were detectable in protoplasts inoculated with either MAM2fGFP or MAM4fGFP (data not shown), viruses encoding MPs with alanine-substitution mutations at positions 92-93 and 121-122, respectively (Bertens et al., 2000). Although occasionally tubular structures could be detected in protoplasts inoculated with MAM2 (Bertens et al., 2000), no tubules were found in protoplasts inoculated with MAM2fGFP. In protoplasts inoculated with two other alanine-substitution mutants, MAM5fGFP and MAM6fGFP, two mutants coding for MPs in which residues 142-143 and 162-163 were changed into alanines, fluorescence was mainly visible in the cytoplasm and in cytoplasmic strands and in the nucleus (Figure 3L and data not shown). These observations indicate that the targeting of these five MP mutants to the cell membrane was affected. Since no tubular structures were visible inside the cytoplasm of the infected protoplasts, it can also be concluded that tubule formation requires targeting of the MP to the cell membrane.

In addition, two mutants with changes in the C-terminal part of the MP were studied. In protoplasts inoculated with MΔA1fGFP, coding for a MP with an insertion of a serine residue between amino acids 262 and 263, strong fluorescence was detectable at the periphery of the cells. The fluorescence accumulated in spots in the cell membrane (Figure 3M) and occasionally, short tubular structures could be detected (data not shown). Faint fluorescence was found in the cytoplasm. Most of the fluorescence found in protoplasts inoculated with deletion mutant M48ΔC3fGFP, which codes for a MP lacking the 59 C-terminal amino acids was distributed over the entire cell membrane and did not accumulate in separate spots in the membrane (Figure 3N).

Next, cowpea leaves were inoculated with some of these mutants and analysed by confocal microscopy at 3 d.p.i.. In epidermal cells of cowpea leaves inoculated with M48ΔAUG2fGFP, the cytoplasm was faintly fluorescent and most of the signal accumulated in the nucleus, as also seen in protoplasts (data not shown). In epidermal cells infected with MAM6fGFP, fluorescent aggregates occasionally containing bright spots were detectable in the cytoplasm near the nucleus. Furthermore, fluorescence was detectable in the cytoplasm and in cytoplasmic strands (Figure 3O). In epidermal cells infected with the C-terminal mutants MΔA1fGFP or M48ΔC3fGFP, fluorescence was present in the nucleus and at the periphery of the cells (Figures 3P and Q). Fluorescent cytoplasmic strands were never observed. At the periphery of the cell, fluorescence was detectable in what seemed to be the cell membranes of two adjacent cells, possibly representing MP:GFP trafficked to neighbouring cells (enlarged in figure 3P and Q). As judged by fluorescence microscopy, about 10-15% of the infection sites on leaves inoculated with mutants MΔA1fGPP and MΔC3fGFP consisted of 2 to 3 cells, whereas with MAM6fGFP, less than 5% of the sites consisted out of more than 1 cell (Table 3).

Table 3: Quantitation GFP-expressing cells in epidermal cells of cowpea plants inoculated with CPMV MP:GFP constructs 12 days after germination

mutant	total no. of infection sites	no. of infected cells 3 d.p.i.		
		1	2	> 2
MPfGFP	48	26 (72%)	6 (17%)	4 (11%)
M19GFP0	68	68 (100%)		
MAM6fGFP	81	80 (99%)	1 (1%)	0 (0%)
MΔA1fGFP	219	200 (91%)	12 (5%)	7 (3%)
MΔC3fGFP	170	150 (88%)	14 (9%)	6 (4%)

Complementation of mutant MPs by wild-type MP

Since none of the mutant MP:GFP proteins described above are able to form tubular structures, it was interesting to investigate if this defect could be removed by the

addition of wild-type MP. Protoplasts were co-inoculated with transcripts of the mutant MP:GFP constructs and transcripts of pTM58SΔ5, that codes for wild-type 58K and MP proteins (Van Bokhoven et al., 1993a). At 48 h.p.i., protoplasts were observed directly by fluorescence microscopy or were fixed and immunostained with anti-MP and a secondary antiserum carrying a cy-3 fluorescent group and subsequently investigated by confocal microscopy. This allowed us to compare the localisation of the mutant MP:GFP fusion (green) with that of all MP present in the cell (MP and MP:GFP; red). In protoplasts inoculated with M58SΔ5 and MΔA1fGFP or MAM2fGFP, long fluorescent tubules were present (Figure 5 A-C and Table 4).

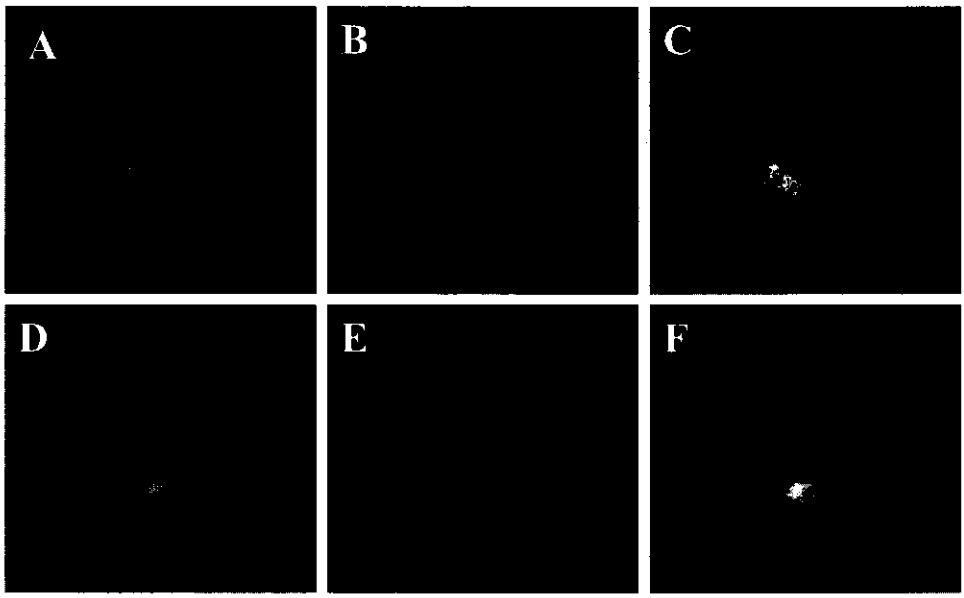

Figure 5:. CLSM images of protoplasts co-inoculated with M58SΔ5 and (A, B, C) MΔA1fGFP or (D, E, F) MAM6fGFP and immunostained with anti-MP. (A, D) green channel showing GFP fluorescence, (B, E) red channel showing cy-3 anti-MP labelling, (C, F) superpositions of (A, B) and (D, E), respectively.

The result suggests that these two mutant MPs still can interact with the wild-type MP. The defect in tubule formation could not be complemented for all MP:GFP mutants (Table 4). For example, after co-inoculation of protoplasts with MAM6fGFP and M58SΔ5, the MP coded by MAM6fGFP was detected in the cytoplasm (green fluorescence in Figure 5D), while the wild-type MP formed tubular structures (Figure 5E). No co-localisation of mutant and wild-type MP was observed (Figure 5F).

Table 4: Complementation of mutant MP:GFP constructs by M5855, which codes for wildtype MP

mutant	tubular structures[1]	fluorescent tubular structures[2]
MΔAUG2fGFP	+	-
MAM2fGFP	+	+ (short)
MAM4fGFP	+	-
MAM6fGFP	+	-
MΔA1fGFP	+	+ (long)

[1] fluorescence of MP in protoplasts immunostained with anti-MP and a cy-3 secondary antibody
[2] fluorescence of MP:GFP in protoplasts

Discussion

In this study we have analysed the subcellular localisation of CPMV derivatives coding for MP:GFP fusion products in living cells. In plants infected with MPfGFP and M8GFP7, MP:GFP fluorescence was mainly detectable as discrete spots in the cell wall. Similar accumulations of MP:GFP have been described for MP:GFP fusion products of alfalfa mosaic virus (AMV) (Huang and Zhang, 1999), TMV (Padgett et al., 1996, Oparka et al., 1997), cucumber mosaic virus (CMV) (Canto and Palukaitis, 1999) beet necrotic yellow vein virus (BNYVV) (Erhardt et al., 2000), and recently apple chlorotic leaf spot virus (ACLSV) (Satoh et al., 2000). In some of these cases, the fluorescent spots have been identified as plasmodesmata, and probably the CPMV MP:GFP protein similarly accumulates in plasmodesmata. Although modified plasmodesmata of CPMV-infected plant cells usually contain long tubular structures (van Lent et al., 1990), such structures were rarely observed in epidermal cells infected with M8GFP7. However, the fluorescent spots observed in these cells probably represent short tubular structures that span only the cell wall. This may be characteristic for epidermal cells, which have a large vacuole and only a thin layer of cytoplasm at the periphery of the cell. Indeed, longer tubular structures have been detected between epidermal and mesophyll cells of plants infected with MPfGFP and M8GFP7.

Except the nucleus and the spots in the cell wall, no clearly defined fluorescent structures like cortical bodies or filaments as found in cells expresing the TMV 30K MP:GFP (Padgett et al., 1996; Reichel and Beachy, 1999) or the AMV P3 MP:GFP (Huang and Zhang,1999) were seen. This has also been reported for a CMV 3a MP:GFP fusion (Canto and Palukaitis, 1999), and these results suggest that the CPMV MP is transported intracellularly via a different mechanism than the TMV MP.

Tubular structures, resembling the tubules present in infected protoplasts, were detected at the upper side of epidermal cells. It is possible that these tubules form at these sites due to the damage caused by the carborundum used for inoculation. This result

subset of protoplasts this MP accumulated in spots in the cell membrane, and occasionally formed short tubular structures. Apparently the MP:GFP coded by this mutant still can, to a certain extend, initiate tubule formation, but is disturbed in tubule elongation. This is supported by co-inoculation experiments with wild-type MP, which shows that mutant MP coded by MΔA1fGFP, which is normally targeted to the periphery of protoplasts and elongated into long tubular structures, can still interact with wild-type MP, while two cytoplasmically localised MP:GFP mutants cannot. In plants, the effect of the mutation in MΔA1fGFP seemed to be more severe than in protoplasts, as the mutant MP:GFP accumulated at several places along the plasma membrane, but was not able to accumulate in spots. Mutant MΔC3fGFP, coding for a MP lacking amino acids 283-342, was likewise targeted to the cell membrane of inoculated protoplasts. However, the MP:GFP coded by this mutant remained scattered all over the plasma membrane and did not accumulate in spots indicating that this mutant apparently is affected in the initiation of tubule formation.

The co-inoculation experiments with wild-type MP suggest that two cytoplasmically localised MP:GFP mutants cannot interact with wild-type MP. However, tubular structures were formed in protoplasts co-inoculated with MAM2fGFP and M58SΔ5. The non-fusion form of this mutant accumulated mainly in the cytoplasm and only occasionally formed tubules (Bertens et al., 2000), but when fused to GFP, this mutant completely lost the ability to form tubules. The observation that this mutant, which has a defect in targeting to the plasma membrane, is still able to interact with wild-type MP, implies that this interaction has to take place in the cytoplasm.

Tripartite viruses consisting of RNA1, MGFPΔCP and MAM2, MAM4, MAM5 or MAM6 can infect whole plants (Bertens et al., 2000). The finding that the cytoplasmically localised MPs coded by MAM4 and MAM6 do not interfere with the spreading of the infection, and thus do not inhibit the activity of the wild-type MP, suggest that the interaction between MP and virions only occurs just before incorporation of the particle in the tubule.

In conclusion, the experiments described in this paper suggest that the region of the CPMV MP between amino acids 262 and 292 is involved in the initial formation of tubular structures on the plasma membrane and the subsequent elongation of these structures. The phenotypes of MΔA1fGFP and MΔC3fGFP suggest that different amino acids might play a role in these two processes. Furthermore, the results described in this paper support the earlier suggestions that the central region of the CPMV MP is involved in intracellular targeting to the plasma membrane and that the presence of a cell membrane is sufficient for tubule formation *in planta*. The process of tubule formation then could be divided into several steps: (1) synthesis of the MP in replication complexes in the cytoplasm close to the nucleus; (2) intracellular transport of MP

molecules to the cell membrane (3) accumulation of MP in spots that leads to the (4) initiation of tubular structures and finally (5) the further elongation of the tubular structures. The modification of plasmodesmata is probably initiated during step (4) and virus particles are probably incorporated in the tubular structure during step (4).

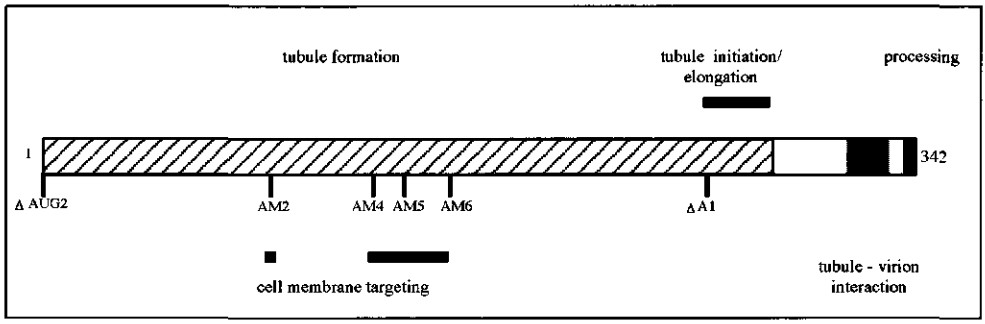

Figure 5:. Schematic representation of the domain structure of the CPMV MP. The MP is shown as a bar. The region of the MP known to be involved in tubule formation is hatched. The regions thought to be necessary for MP-virus particle interaction and proteolytic processing are shown as a grey and black bar respectively. Residues involved in cell membrane targeting and tubule initiation/elongation are indicated below and above the MP bar, respectively.

Materials and methods

Generation of CPMV mutants

All routine DNA manipulations were performed according to Sambrook et al. (1989). Enzymes used were purchased from Life Technologies (Eggenstein, Germany) or Boehringer Mannheim (Mannheim, Germany). Plasmids pTB1G and pTM1G that contain a full-length clone of RNA1 or RNA2 respectively, from which infectious transcripts can be obtained by *in vitro* transcription, have been described previously (Eggen et al., 1989). In all GFP-containing constructs the plant optimised GFP5 gene (Siemering et al., 1996), in which the *Cla*I site had been mutagenised, was used (Gopinath et al., 2000). The construction of plasmid pM19GFP7, in which the GFP5 gene is introduced in RNA2 flanked by two coding regions for artificial cleavage sites, has been described by Gopinath et al. (2000).

For construction of pMPfGFP, a 1.5 kb RNA2 fragment was obtained by polymerase chain reaction using primers M44$^+$ (5'-ACCCGAACCAAACCTTCTTC-3') and M1540E$^-$ (5'-CGGGAATTCCATTTGTGGAAAAGCCACATTCCC-3'). This fragment was digested

with *Bgl*II (nt 189) and *Eco*RI (underlined in primer 11540E⁻) and ligated to a 5.0 kb *Bgl*II-*Eco*RI fragment of pTM19GFP7 (Gopinath et al., 2000).

RNA2 constructs coding for fusions of mutant MPs and GFP were constructed by digestion of pTMΔAUG2 (Wellink et al., 1993b), pTMAM2, pTMAM4, pTMAM5, pTMAM6 (all described in Bertens et al., 2000) and pTMΔA1 (Lekkerkerker et al., 1996) with *Nar*I (nt 214) and *Bam*HI (nt 1504). The 1.3 kb fragments were cloned into pTMPfGFP digested with the same restriction enzymes to obtain pTMΔAUG2fGFP, pTMAM2fGFP, pTMAM4fGFP, pTMAM5fGFP, pTMAM6fGFP and pTMΔA1fGFP. For construction of pTMΔC3fGFP, a 1.3 kb fragment was obtained from pTM1G using primers M44⁺ and M1363⁻ (5'-GGAATTCAGCATTGCAGGAGTTGGATAAC-3'). This PCR-product was digested with *Bgl*II (nt 189 in RNA2) and *Eco*RI (underlined in primer M1363⁻) and cloned into pTMPfGFP digested with *Bgl*II and *Eco*RI.

Plasmid pTMPfGFPΔCP was constructed by digestion of pTMPfGFP with *Xba*I (present at the 3' site of GFP) and *Nae*I (3173). The 4.9 kb fragment was isolated from gel, end-filled with Klenov, and religated. Plasmid pTM19GFPΔCP was constructed similarly, using pTM19GFP7 (Gopinath et al., 2000) as a template.

Inoculation of plants and protoplasts

Cowpea (*Vigna unguiculata* cv. California Blackeye) and *Nicotiana benthamiana* plants were grown as previously described (Van Bokhoven et al., 1993b). Viral RNAs were transcribed from full-length cDNA clones as described by Van Bokhoven et al (1993b), except that DTT was included in each reaction to a final concentration of 0.01M. Inocula were prepared from protoplast samples or plant tissue by grinding the tissue in phosphate buffer (PBS: 100 µM sodium phosphate, 1.5 M NaCl, pH 7.2). Cowpea mesophyll protoplasts were isolated and inoculated with transcripts or protoplast or plant extracts as described by Van Bokhoven et al. (1993b). After inoculation, protoplasts were kept under continuous illumination at 25 °C. Protoplasts were collected by centrifugation at 3,000 rpm and stored at - 80°C until further use. Inocula were made from these protoplasts by resuspending the frozen pellets in 50 µl PBS and homogenisation by straining of the protoplasts through a 0.45 mm syringe for 10-15 times.

Protein analysis

Collected protoplasts were extracted in buffer (50 mM Tris-HAc, pH 7.4, 10 mM Kac, 1 mM EDTA, 5 mM DTT, 0.5 mM PMSF). Cytoplasmic and membrane-associated fractions were separated as described before (Bertens et al., 2000). SDS-polyacrylamide gel electrophoresis on 12.5 % gels and electroblotting was performed as described by Gopinath et al. (2000). For immunostaining , blots were blocked with 5% nonfat dry

milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) and then incubated with polyclonal antiserum raised against the 58K/MP proteins (with a dilution of 1:10000) in TBST containing 0.5% nonfat dry milk for 1 hr. After three washes with TBST, the membranes were incubated for 1 hr in TBST containing 1:5000 diluted anti-IgG-alkaline-phosphatase. Finally, the membranes were washed in TBST, TBS, and alkaline-phosphatase buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) and developed using nitro blue tetrazolium (Life Technologies) and 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies) as substrates.

Detection of GFP fluorescence

Fluorescence in living protoplasts or leave pieces was observed using an epifluorescence microscope (Nikon Optiphot 2) equipped with an FITC filter set or a Zeiss LSM510 confocal microscope. Excitation was obtained with the 488 nm line of an argon ion laser. Fluorescence of GFP was observed using a cutoff filter passing light of wavelength above 505 nm. For image recording, we typically used an X40 plan Neofluor objective (NA0.75; Zeiss) and a 488/543 nm dual dichroic excitation mirror with a 510-540 nm emission filter. Images from the confocal microscope were processed using Adobe Photoshop 5.0 software (Adobe Systems Inc., Mountain View, CA). The infections in cowpea and *N. benthamiana* plants were also observed using a Black Ray long-wave ultraviolet lamp (B100AB; Ultraviolet Products).

Immunofluorescence

For immunostaining, protoplasts were spotted on glass slides coated with poly-L-lysine (0.05% w/v) and left for 5 min. Then, the samples were fixed by immersion in 96% ethanol for 20 minutes. After thorough washing (2 times for 15 min in PBS and an incubation in PBS supplemented with 4% bovine serum albumine (BSA) to block non-specific antibody binding places, the samples were incubated for 1 hr with anti-MP (diluted 1:250 in PBS supplemented with 1% BSA). Subsequently, the samples were washed again with PBS (3 times for 15 min.) and incubated with a secondary antiserum (goat-anti-rabbit-cy3 (Sigma) diluted 1:60 in PBS-1% BSA) for 1 hr. Samples were investigated under the confocal microscope.

Electron microscopy

Copper grids (400-mesh) were discharged and coated with poly-L-lysine (0.05%). Protoplasts were placed on these grids and incubated for 3 min. After washing with

double-distilled water, theprotoplasts were fixed with 2% (w/v) phosphotungstic acid (PTA) in double distilled water, pH 5.5. Excess PTA was removed with filter paper, and the samples were examined after drying under a Philips CM12 electron microscope.

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Chapter 5

CONCLUDING REMARKS

The aim of the research described in this thesis was to investigate the cell-to-cell movement of cowpea mosaic virus (CPMV) with special emphasis on the role of the 48K viral movement protein (MP). In chapter 2, the effect of subtle mutations in the MP sequence, obtained by alanine scanning mutagenesis, on the subcellular distribution of the MP, tubule formation and infectivity in plants was investigated. This analysis was extended in chapters 3 and 4. In chapter 3, efforts were undertaken to delineate the precise C-terminal border of the MP domain involved in tubule formation. We also studied the role of the C-terminus of the MP in viral infection using tripartite viruses and viruses coding for MP:GFP (green fluorescent protein) fusion proteins. In chapter 4, emphasis was put on the analysis of the subcellular localisation of wild-type and mutant MP:GFP products in living cells.

The results of these experiments clearly indicate that the 48K MP can be divided into two regions, each with a different role in the movement process of CPMV from cell-to-cell. A large region, encompassing amino acids 1-292, is absolutely necessary for tubule formation, while the C-terminal 50 amino acids are not involved in tubule formation, but comprise a domain necessary for the incorporation of virus particles into the tubular structures. Since mutants lacking up to 50 C-terminal amino acids are able to form tubular structures in protoplasts, the structures of the MP necessary for the intracellular transport of the MP to the plasmodesmata and possibly involved in an interaction with host factors preceding the initiation of tubule formation, are as well present within the first 292 amino acids.

The large domain necessary for tubule formation can be subdivided into smaller regions with additional specific roles. Mutations in the N-terminal and central parts of the tubule-forming domain often prevent correct targeting of the MP (chapter 4). Whereas in plant cells infected with wild-type virus, the 48K MP is directed to the plasma membrane to form tubular structures, the MPs produced by such mutants are found throughout the cytoplasm. Mutant MP in which amino acids 92 and 93, representing the LPL-domain conserved in MPs of different viruses, were altered, had lost its tubule forming capacity and the MP remained localised in the cytoplasm, as was clear from our MP:GFP fusion studies. A mutant in which the amino acids 162 and 163 were

substituted by alanine residues mostly accumulated near the cytoplasmic membrane vesicles which are the sites of virus replication, and slowly dispersed into the cytoplasm. This mutation appeared to result in aggregation of the MP and the mutant MP is unable to find its way to the plasmodesmata. Substitution mutants with alanine residues at the positions 121-122 or 142-143 similarly remained in the cytoplasm, but did not show the strong accumulation at the sites of virus replication.

The exact mechanism by which the CPMV MP is transported to plasmodesmata is unknown. It is possible that the MP moves intracellularly via the cytoskeleton, or finds its way associated with elements of the endoplasmic reticulum (ER), two cellular pathways suggested to play a role in targeting of other viral MPs to plasmodesmata (see chapter 1). So far, our investigations of plants and protoplasts inoculated with CPMV variants that code for MP:GFP fusion proteins did not reveal any evidence for a direct association of the MP to either the cytoskeleton or the ER (chapter 4). However, this association might be transient, or occur during stages of the infection other than studied here. It would be interesting therefore, to test the effects of components that disrupt the structure of the cytoskeleton and ER on MP targeting and tubule formation.

A remarkable result has come from experiments showing that in the presence of wild-type MP, the behavior of some mutant MPs can be changed (chapter 4). For these experiments we have very profitably used tripartite viruses in which one genome segment coded for mutant MP:GFP and an other genome segment for wild-type MP. Such experiments revealed that MPs in which amino acids 92 and 93 were changed for alanine residues were incorporated into tubular structures in the presence of wild-type MP. These observations show that in the presence of wild-type MP, the mutant MPs can be targeted to the sites of tubule formation and it seems that wild-type MP can restore the defect in the targeting of the mutant MP. If indeed the wild-type MP can guide mutant MP to the site of tubule formation and achieve incorporation of mutant MP in tubules, this may suggest that perhaps MP dimers, in which one part can be mutant MP, are the form in which MP is carried from the sites of synthesis in the cytoplasm to the plasmodesmata. Not all mutant MPs show restoration of behavior by mixing with wild-type MP. Mutants coding for MPs in which amino acids 121-122 or 162-163 were changed for alanine residues, or a mutant MP lacking the N-terminal four amino acids, were not found in tubular structures in the presence of wild-type MP, suggesting that these MPs cannot form dimers with wildtype MP.

Mutant MPs with alterations near the C-terminus of the tubule-forming domain (between amino acids 262 and 292) are targeted to the periphery of protoplasts, but either remain

scattered all over the cell membrane or accumulate in spots in the membrane and occasionally in short tubules, unable to elongate into longer structures. The short tubules are not able to translocate the virus into a neighbouring cell. These results suggest that the spots represent the sites for the initiation of the tubular structures.

The C-terminal part of the MP is important for incorporation of virus particles into the tubular structure. This process is disturbed in viruses coding for MP lacking amino acids 314-332 or MP in which the last 10 amino acids have been replaced by GFP (chapter 3) and in viruses coding for MP fused to GFP at its C-terminus (chapter 4). Infections of such mutant viruses remain restricted to single epidermal cells and in infected protoplasts only empty tubules are formed. MP with a deletion in the C-terminal domain involved in the incorporation of virus particles in tubules could be complemented by wild-type MP *in planta* as well as in protoplasts. Again the tripartite virus proved to be of great value for these experiments (chapter 3). In plants the complementation had the effect that the infection was no longer restricted to single epidermal cells but normally spread through the plants. In protoplasts the tripartite complementing virus indeed formed tubular structures containing virus particles, which is in agreement with the infectious behavior in plants, but the virus particles were not nicely virion-to-virion arranged like in tubules produced by wild-type virus. These results argue in favour for a specific interaction between virus particles and the C-terminus of the MP and strongly suggest that virus particles are incorporated in the tubules during the process of tubule formation. Presumably the tubules formed by the tripartite complementary virus contain both wild-type MP and MP deleted in the C-terminal end unable to form filled tubules by itself. The mutant MP which is defective in the specific interaction with virus particles forms tubular structures that contain disorderly arranged virions, and tubules with fewer particles as virions are only incorporated by interaction with wild-type MP.
We have tried to investigate the specificity of the interaction between the C-terminal end of the MP and the virus particles also by constructing hybrid MPs containing the tubule-forming domain of red clover mottle virus (RCMV) MP and the C-terminal residues of CPMV MP. RCMV and CPMV are related comoviruses, but the C-terminal ends of the MPs of both viruses show only limited amino acid sequence homology. One such hybrid MP was able to form tubular structures in cowpea protoplasts, but unfortunately we were not able to determine whether the hybrid MP allowed incorporation of virions into tubules, as the viruses coding for hybrid MPs replicated very poorly (chapter 3). In itself, the poor replication was an unexpected result. Previously we had demonstrated that the 10K N-terminal part of the 58K protein is required for the proper replication of CPMV RNA2 (Van Bokhoven et al., 1993a).

Mutants which code for 48K protein only and do not produce the 58K protein with the additional 10K, are not able to replicate. Also the tripartite virus functions only if a 10K is encoded on the RNAs, whereas the 48K coding sequence can be deleted. The defective replication of the RNA2 encoding a hybrid RCMV/CPMV MP indicates that the presence of 10K only is no sufficient guarantee for replication. In this case, the poor replication may be attributed to interactions of the 10K protein part with the foreign protein sequence encoded by the RNA2, preventing proper functioning of 10K in replication.

Electron microscopical examination of CPMV-infected plants suggest that the modified plasmodesmata containing tubular structures lack a desmotubule (van Lent et al., 1990). This raises the question whether the CPMV MP actively removes the desmotubule. The mechanism underlying such a process is unknown, but, as the desmotubule is a component of the ER, may involve interactions between the viral MP and specific ER proteins that lead to removal of the desmotubule before or concomitantly with tubule formation. Alternatively the viral MP may initiate the formation of new intercellular connections, as suggested by Van der Wel et al. (2000) to occur during infections of alfalfa mosaic virus (AMV). The number of plasmodesmata present in cells at the border of the AMV infection apparently is higher than in tissue in which the AMV infection is fully established or in uninfected tissue. In chapter 4 we have described how tubular structures resembling tubules present in infected protoplasts were detected at the upper sites of the epidermal cells and we have argued that tubule formation in plants, as in protoplasts, does not require the presence of functional plasmodesmata. Also, we have shown that MP accumulates in spots in the cell wall between epidermal cells and the guard cells of stomata, which do not contain plasmodesmata. Such observations support the idea that the tubules are not necessarily formed through existing plasmodesmata but that new intercellular channels may be formed during CPMV infection.
The tubular structures, formed by the CPMV MP during viral infection of plants, protrude from the entrance of a plasmodesma in an infected cell through the cell wall into the cytoplasm of a neighbouring cell (Van Lent et al., 1990). Whatever the beginning of tubule formation is, a tubule filled with virus particles runs from the entry of a plasmodesma through the plasmodesma into a neighbouring cell. This strongly suggest a specific site for initation of tubule formation and assembly of MPs into tubular structures filled with virions. Presumably then the initation site is also defined by one or more specific host proteins. Attempts to isolate such a host factor using the yeast two-hybrid system have so far been unsuccessful (Jongejan and Bol, personal communication). Biochemical analysis of tubular structures isolated from CPMV-infected protoplasts does not indicate the presence of host proteins and makes it

unlikely that such a protein is present next to the MP in stochiometric ratios. Furthermore, it is interesting to know how the formation of tubular structures at other sites in the cell is prevented. The CPMV MP might recruit cellular chaperonins that prevent mature folding of the MP into a form capable of initiating tubule growth.
Tubule formation has profound effects on the morphology of the host cell. In plants, the tubules penetrate the cell wall through modified plasmodesmata, while in protoplasts, the tubules extend from the cell surface into the incubation medium surrounded by cell membrane. It is likely that both processes require considerable amounts of energy, but little or no attention has been given to the energetic aspects of the assembly of tubular structures and the cell-to-cell movement of viruses. It will be of great interest to prepare the MP in a way which allows to study the biochemistry and structure of the MP in more detail. So far there is no indication that the virus particles move through the tubular structures. At the other hand, it seems feasible that the virus particles are incorporated in the tubular structure during tubule formation and are subsequently released in the neighbouring cell when the tubule has reached so far.

The results described in this thesis suggest the following model for cell-to-cell movement of CPMV. After entry into an uninfected cell, the virus will start translation and replication of its genome. Viral replication is associated with small membraneous vesicles originating from the ER. After synthesis, the MP is transported towards the cell membrane via an yet unknown mechanism and accumulates in larger spots in the cell membrane. Since these spots are the initiation site for tubule formation, they may be located near plasmodesmata. This accumulation initiates tubule formation and the modification of plasmodesmata. Virus particles present near the tubule-initiation site will be recruited and incorporated into the tubule during its elongation. The tubule grows from its site of synthesis unidirectionally across the cell wall into a neigbouring cell, where the tubule desintegrates via an unknown mechanism, releasing the viral particles.

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NEDERLANDSE SAMENVATTING

Virussen zijn subcellulaire parasieten die niet zelfstandig kunnen bestaan, maar cellen binnendringen en cellulaire mechanismen van hun gastheer gebruiken voor vermenigvuldiging. Virussen zijn verantwoordelijk voor vele ernstige ziektes bij mensen en dieren (zoals bijvoorbeeld griep, verkoudheid of AIDS), maar ook bij planten, waar virussen verantwoordelijk kunnen zijn voor de vernietiging van hele oogsten. Virussen zijn vrij eenvouding van structuur en bevatten een kleine hoeveelheid genetisch materiaal, dat uit DNA of RNA kan bestaan. Dit genoom is omgeven door een beschermende eiwitlaag, de mantel.

Terwijl dierlijke virussen normaal cellen binnenkomen via chemische interacties met specifieke herkenningsmoleculen (receptoren) in de celmembraan, zijn celmembranen van plantencellen omhuld door een pantserbekleding van cellulose die een ondoordringbare hindernis vormt voor plantenvirussen. De meeste virussen kunnen een plant pas binnendringen na mechanische beschadiging of met behulp van een biologische vector (b.v. een bladluis of een ander insect) die de virussen tijdens het voeden rechtstreeks in een gastheercel binnenbrengen. In zo een initiëel geïnfecteerde cel wordt het virale genoom, dat uit één of meerdere DNA of RNA moleculen kan bestaan, vertaald in virale eiwitten die zorg dragen voor de replicatie van het virale genoom en de bemanteling van het virale RNA. Na deze vermenigvuldigingsstap verspreidt het virus zich verder door de plant en zal in eerste instantie de naburige cellen infecteren (via het zogeheten lokaal transport of cel-cel transport). Nadat een infectie de vaatbundels bereikt heeft, zullen virusdeeltjes zich via de vaatbundels door de rest van de plant verspreiden (het lange-afstands transport). In de hoger gelegen bladeren treedt het virus de vaatbundels weer uit, en verspreidt het zich opnieuw via cel-cel transport naar andere cellen van dat blad.

Als een virus zich vanuit een initiëel geïnfecteerde cel naar naburige cellen wil verspreiden, stuit het opnieuw op de ondoordringbare celwand. Deze hindernis wordt met behulp van een speciaal mechanisme omzeilt. In de celwand zitten nauwe kanaaltjes, de plasmodesmata, die naburige cellen met elkaar verbinden. Deze

kanaaltjes spelen een rol bij de verspreiding van (voedings)stoffen en worden door de plant gebruikt voor intercellulaire communicatie. Virussen zouden deze plasmodesmata kunnen gebruiken voor lokaal transport, ware het niet dat ze te groot zijn om door de kanaaltjes heen te kunnen gaan. De oplossing die virussen hebben ontwikkeld om dit probleem te omzeilen is even elegant als simpel: ze coderen voor een specifiek eiwit (het transporteiwit, afgekort MP voor het Engelse "movement protein"), die de diameter van plasmodesmata kan vergroten, waardoor transport van virusdeeltjes of complexen van viraal RNA en MP mogelijk wordt. In **hoofdstuk 1** wordt een overzicht gegeven van virale MPs en de verschillende mechanismen die plantenvirussen hebben ontwikkeld om zich van cel naar cel te verspreiden.

Dit proefschrift beschrijft een onderzoek waarin het MP van het koebonenmozaiekvirus (op z'n Engels "cowpea mosaic virus" (CPMV) geheten) centraal staat. Dit virus infecteert voornamelijk tropische planten, waaronder kouseband (*Vigna unguiculata*), een plant waarvan de peulen vooral in Afrika en Zuid-Amerika een belangrijke voedingsmiddel zijn (kouseband is ook in Nederland op veel plaatsen te koop; voor een tip hoe deze te bereiden, kan de auteur van dit proefschrift benaderd worden). Een infectie van kouseband door CPMV wordt gekenmerkt door de vorming van milde tot hevige mozaiek-symptomen op geïnfecteerde bladeren, die tot afsterven van het blad kan leiden. Het genoom van CPMV bestaat uit een tweetal enkelstrengs RNA moleculen met een positieve polariteit. Deze RNA moleculen worden in een geïnfecteerde cel vertaald in een drietal polyeiwitten, die door een viraal enzym in stukken worden geknipt tot kleinere producten. De door het RNA1 gecodeerde eiwitten zijn betrokken bij de virale replicatie. De eiwitten die gecodeerd worden door RNA2, spelen een rol bij het cel-cel en het lange-afstands transport van CPMV. Dit zijn de twee manteleiwitten LCP en SCP, een 58K eiwit, dat nodig is voor replicatie van RNA2 en het MP.

Met behulp van electronen-microscopische analyse zijn in CPMV-geïnfecteerd weefsel buisvormige structuren aangetroffen, die gevuld zijn met virusdeeltjes. Deze buizen steken door een gemodificeerde plasmodesma van een geïnfecteerde cel in het cytoplasma van een naburige cel. Soortelijke structuren zijn aangetroffen in protoplasten (geïsoleerde plantencellen) die geïnfecteerd zijn met CPMV en in protoplasten of insectencellijnen waarin alleen het MP specifiek tot expressie is gebracht. Deze experimenten laten zien dat voor de vorming van de buizen geen plasmodesmata nodig zijn. Biochemische analyse van de buizen heeft laten ziendat de buizen grotendeels of zelfs helemaal zijn opgebouwd uit het MP.

Het proces van buisvorming gebeurd waarschijnlijk in een aantal stappen. Nadat het MP aangemaakt is in virale replicatie complexen moet het naar de celmembraan getransporteerd worden, waar het zich bij plasmodesmata zal ophopen en een buis zal vormen. Tijdens deze buisvorming worden de virusdeeltjes in de buis ingebouwd. De plasmodesma waarin de buis wordt gevormd, wordt drastisch gemodificeerd. Verschillende gebieden (domeinen) van het MP zullen een specifieke rol hebben en betrokken zijn bij een of meerdere van de hierboven beschreven stappen. In **hoofdstuk 2** hebben we getracht zulke domeinen te vinden door met behulp van moleculair-biologische technieken mutaties aan te brengen binnen het MP en het effect hiervan op onder andere de buisvorming te onderzoeken. Uit de resultaten blijkt dat het resultaat van een subtiele mutatie heel drastisch kan zijn. Sommige mutaties hebben tot gevolg dat het MP geen buizen meer kan vormen. Virussen die dit mutant MP bezitten zijn niet meer in staat om zich te verspreiden in planten. Mutaties aangebracht op andere plaatsen in het MP lijken nauwelijks effect te hebben op de eigenschappen van het transporteiwit. We hebben ontdekt dat het N-terminale en het centrale deel van het MP nodig zijn voor het transport naar de plasmodesmata en voor de vorming van de buizen.

Het volgen van een virusinfectie in levende planten is erg moelijk. Virussen zijn alleen maar te zien onder een electronenmicroscoop waarvoor men echter de delen van de plant die men wil onderzoeken moet fixeren. Door deze behandeling sterven de plantcellen en kunnen er allerlei ongewilde veranderingen (artefacten) optreden in het weefsel. Een paar jaar terug is er een nieuwe methode ontwikkeld om een virusinfectie in levende planten te volgen. Men maakt hiervoor gebruik van een fluorescent eiwit, het "green fluorescent protein" (GFP). Dit eiwit komt van nature voor in kwallen en heeft de bijzondere eigenschap om groen op te lichten (te fluoresceren) indien het wordt beschenen met UV-licht. Met behulp van moleculair-biologische technieken is dit GFP ingebouwd in CPMV. Als we planten hiermee infecteren, kunnen we de infectie in de tijd volgen door de planten onder een UV-lamp of onder een fluorescentie-microscoop te onderzoeken, zoals staat beschreven in de hoofdstukken 3 en 4.

In **hoofdstuk 3** hebben we ons geconcentreerd op het C-terminale deel van het MP. Door de analyse van deletie-mutanten en hybride MPs (bestaande uit delen van het MP van het rode-klavervlekkenvirus en dat van CPMV) was het mogelijk om bijna op het aminozuur nauwkeurig de grens aan te geven van het domein dat betrokken is bij de

vorming van buizen. De buizen gevormd door een MP mutant, waarin de laatste 10 aminozuren waren vervangen door GFP, bleken geen virusdeeltjes te bevatten. Blijkbaar blokkeert GFP het functioneren van een MP domein dat betrokken is bij de inbouw van virusdeeltjes in buizen. Indien een virus naast wild-type (normaal) MP ook een mutant MP (die geen functionele C-terminus heeft) bezit, verspreidt het virus zich toch door de plant. De buizen, die waarschijnlijk zowel uit het wild-type alsook het mutant MP bestaan, zijn niet helemaal netjes gevuld met virusdeeltjes. Af en toe lijkt er wat ruimte tussen de verschillende deeltjes te zitten. Deze resultaten geven aan dat alle C-termini nodig zijn voor het virale transport.

Een van de bijzondere eigenschappen van GFP is, dat na fusie van GFP aan een ander eiwit (b.v. het MP), de intracellulaire localisatie van dat eiwit kan worden bekeken in levend materiaal. In **hoofdstuk 4** hebben we de intracellulaire locatie van een aantal fusieproducten tussen GFP en het CPMV MP nader onderzocht. In eerste instantie hebben we een fusie gemaakt tussen het wild-type MP en GFP. Een virus coderend voor dit MP:GFP fusieproduct was in staat (fluorescente) buizen te maken in protoplasten. In planten hoopt het MP:GFP zich op als opvallende punten (spots) in de celwand. Deze plaatsen zijn waarschijnlijk de eerder aangehaalde gemodificeerde plasmodesmata, waar cel-cel verspreiding van het virus plaatsvindt. In sommige gevallen werden zelfs fluorescente buizen in de celwand aangetroffen. De infectie van dit genetisch gemodificeerde virus bleek echter beperkt tot enkele cellen. Vervolgens zijn een aantal van de in hoofdstuk 2 gekarakteriseerde MP mutanten gefuseerd aan GFP en is de localisatie van deze mutant MP:GFP eiwitten bekeken in protoplasten en planten. MPs die een mutatie bevatten in hun centrale deel bleken verstoord te zijn in het intracellulaire transport naar de celmembraan en hoopten zich op in het cytoplasma. Een van de mutant MP:GFPs hoopte zich tevens op in structuren die in de buurt van de celkern in het cytoplasma liggen. Waarschijnlijk zijn dit virale replicatie-complexen en is dit mutant MP:GFP niet in staat zich hieruit los te maken. Mutante MP:GFPs met veranderingen in het C-terminale deel van het MP werden wel naar de celmembraan getransporteerd, maar waren verstoord in de initiatie of elongatie van de buizen.

We kunnen concluderen dat het in dit proefschrift beschreven onderzoek heeft geleid tot een beter inzicht in het mechanisme dat CPMV gebruikt voor zijn cel-cel verspreiding. Middels mutatie-analyse en door gebruik te maken van GFP hebben we een aantal functionele domeinen van het CPMV MP geïdentificeerd. We kunnen het MP grofweg in twee functionele domeinen verdelen. Een groot deel van de N-terminale

en centrale gebieden van het eiwit zijn betrokken bij de vorming van buisvormige structuren in protoplasten en planten. Dit domein is onder te verdelen in gebieden die betrokken zijn bij het intracellulaire transport naar de plasmodesmata toe en in gebieden die een rol spelen bij de initiatie van buisvorming. Het C-terminale deel is niet betrokken bij buisvorming, maar speelt een rol bij de inbouw van virusdeeltjes in de buizen. De in dit proefschrift beschreven resultaten zullen worden gebruikt voor vervolgstudies, die onder andere gericht zullen zijn op de identificatie van gastheer-eiwitten die een interactie aangaan met het CPMV MP en op het ophelderen van het intracellulaire transport van het MP.

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Iedereen bedankt!

Peter

CURRICULUM VITAE

Op 1 augustus 1969 werd ik, Petrus Joannes Alexander Bertens, geboren in Nijmegen. Na het behalen van het VWO diploma aan de Rijksscholengemeenschap 't Slingerbos te Harderwijk, begon ik in september 1987 aan een studie Biologie aan de Universiteit Utrecht. Tijdens deze studie heb in het kader van een afstudeervak onderzoek gedaan naar de cholesterol afgifte door de middendarm naar de hemolymfe van de Afrikaanse treksprinkhaan *Locusta migratoria*. Dit gebeurde onder begeleiding van Prof. Dr. Dick van der Horst, Prof. Dr. Ad Beenakkers (Projectgroep Stofwisselingsfysiologie, Vakgroep Experimentele Dierkunde, Universiteit Utrecht) en Prof. Dr. Hari Agarwal (University of Delhi, India. Een tweede afstudeervak heb ik uitgevoerd op het Nederlands Instituut voor Ontwikkelingsbiologie te Utrecht, alwaar ik onder begeleiding van Dr. Gauko Kuiken en Dr. Olivier Destrée de promoter isoleerde van het *Xenopus laevis* (Afrikaanse Klauwpad) *Xwnt*-5C proto-oncogen. Na het behalen van mijn doctoraaldiploma in januari 1994 heb ik van mei 1994 tot april 1995 mijn militaire dienstplicht vervuld als sergeant op het Opleidingscentrum Militair Geneeskundige Dienst te Hollandse Rading, waar ik betrokken was bij de militaire opleiding van andere dienstplichtigen. Van september 1995 tot augustus 1999 heb ik gewerkt als onderzoeker-in-opleiding in de "CPMV groep" van het Laboratorium voor Moleculaire Biologie van Wageningen Universiteit, onder begeleiding van Dr. Joan Wellink en Prof. Dr. Ab van Kammen. De resultaten van dat onderzoek staan beschreven in dit proefschrift. Tegenwoordig ben ik beleidsmedewerker bij de Nederlandse Biotechnologie Associatie (Niaba) in Leidschendam.