Interaction between the *Alfalfa mosaic virus* movement protein and plasmodesmata

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. Gedurende de infectie van plantenweefsel produceert luzernemozaïekvirus transportbuizen ten behoeve van cel-tot-cel verspreiding (dit proefschrift).

. Tijdens het cel-tot-cel transport van luzernemozaïekvirus neemt het aantal plasmodesmata aan het infectie-front tijdelijk toe (dit proefschrift).

. De waarneming dat op de grens van infectie het manteleiwit van tabaksmozaïekvirus in de plasmodesmata aanwezig is, is te opmerkelijk om niet verder onderzocht te worden. (Tomenius *et al.*, 1987, Virology 160, 363-371).

. Het werk van Ehlers *et al.*, (1996) weerlegt het dogma dat protoplasten geen plasmodesmata bezitten. (Planta 199, 126-151).

5. De lipiden uit de celwand van Mycobacteriën zijn cruciaal voor de ontwikkeling van een vaccin tegen deze ziektewerkers. (Beckman *et al.*, 1994, Nature 372, 691-694, Sieling *et al.,* 1995, Science 269, 227-230)

6. Combinatie van de grote hoeveelheid celbiologische gegevens met de huidige informatietechnologie, zal het blikveld van de wetenschapper verbreden.

7. Indien inter- en intracellulair transport van virussen met een zelfde efficiëntie zou verlopen als inter- en intrastedelijk transport van mensen, dan zou de economische schade van virusinfecties veel kleiner zijn.

8. Teveel flexibiliteit kan leiden tot verstarring.

9. Perfectie wordt zelden bereikt door menselijk perfectionisme.

Stellingen behorend bij het proefschrift:

Interaction between the *Alfalfa mosaic virus* movement protein and plasmodesmata

Wageningen, 20 september 2000,

N.N. van der Wel.

Hoe meer je weet,
des te beter je weet,
dat je weinig weet.

Contents

Chapter 1

General introduction

Plant viruses are intracellular parasites which utilise various host mechanisms to fulfill a complete infection cycle. To establish intercellular translocation throughout the whole plant, viruses exploit plasmodesmata, the intercellular communication channels of the plant. These complex pores through the compact cell wall create an intercellular continuum that allows neighbouring plant cells to communicate and to regulate intercellular transport. In mature leaves, plasmodesmata of mesophyll cells only allow passage of small (<1 kDa) molecules. Since viral genomes and virus particles are much larger, viruses need mechanisms to modify these channels in such a way that these entities can be translocated to neighbouring cells. For this purpose, plant-infecting viruses encode specialised proteins referred to as movement proteins (MPs) that are involved in the modification of the plasmodesmata to achieve cell-cell movement of the virus in various forms.

Since viral MPs interact specifically with plasmodesmata the study of cell-to-cell movement of plant viruses may also shed light on cell-cell communication and intercellular transport of macromolecules and signals in higher plants in general. Therefore, in 1995 a programme, subsidised by the Council for Earth and Life Sciences (ALW) of the Netherlands Organisation for Scientific Research (NWO) was initiated, of which the studies described in this thesis were made part of, and aimed at the elucidation of the role of plasmodesmata in cell-cell communication and in virus transport. This research programme encompassed three PhD research lines which all centred around the unravelling of the role of plasmodesmata in transport of two distinct viruses, *Alfalfa mosaic virus* (AMV) and *Cowpea mosaic virus* (CPMV), in their common host, *Nicotiana benthamiana.* The rationale behind the choice of these two plant viruses was that at the onset of the programme the consensus was that AMV would move in the "tobamovirus-like" fashion, i.e. in a non-virion form, through minor modified plasmodesmata, while CPMV was known to use a tubule-guided movement mechanism. Hence, the MPs of both viruses would accordingly associate with plasmodesmata in very distinct ways, thereby representing two distinct molecular "probes" for MP-plasmodesmata binding studies. The PhD theses of the research programme describe the identification and characterisation of host proteins that specifically interact with viral proteins, using the yeast two hybrid system (PhD thesis of L. Jongejan, Leiden University), and the mapping of functional domains of the MPs of AMV and CPMV studied by site-directed mutagenesis (PhD thesis of P. Bertens, Wageningen University). The current thesis focuses on the *in situ* analysis of the MP of AMV and its interaction with plasmodesmal and possibly other host proteins.

Before presenting and discussing the experimental data obtained during this research project, first an overview will be given on what is known about the structure, composition, and functioning of plasmodesmata and about AMV and its MP.

Plasmodesmata

Functioning of plasmodesmata

In plants individual cells are surrounded by a cell wall which stabilises the cell configuration and allows plant cells to function in the hypertonic environment of the plant. The cell wall creates a physical barrier between neighbouring cells although the
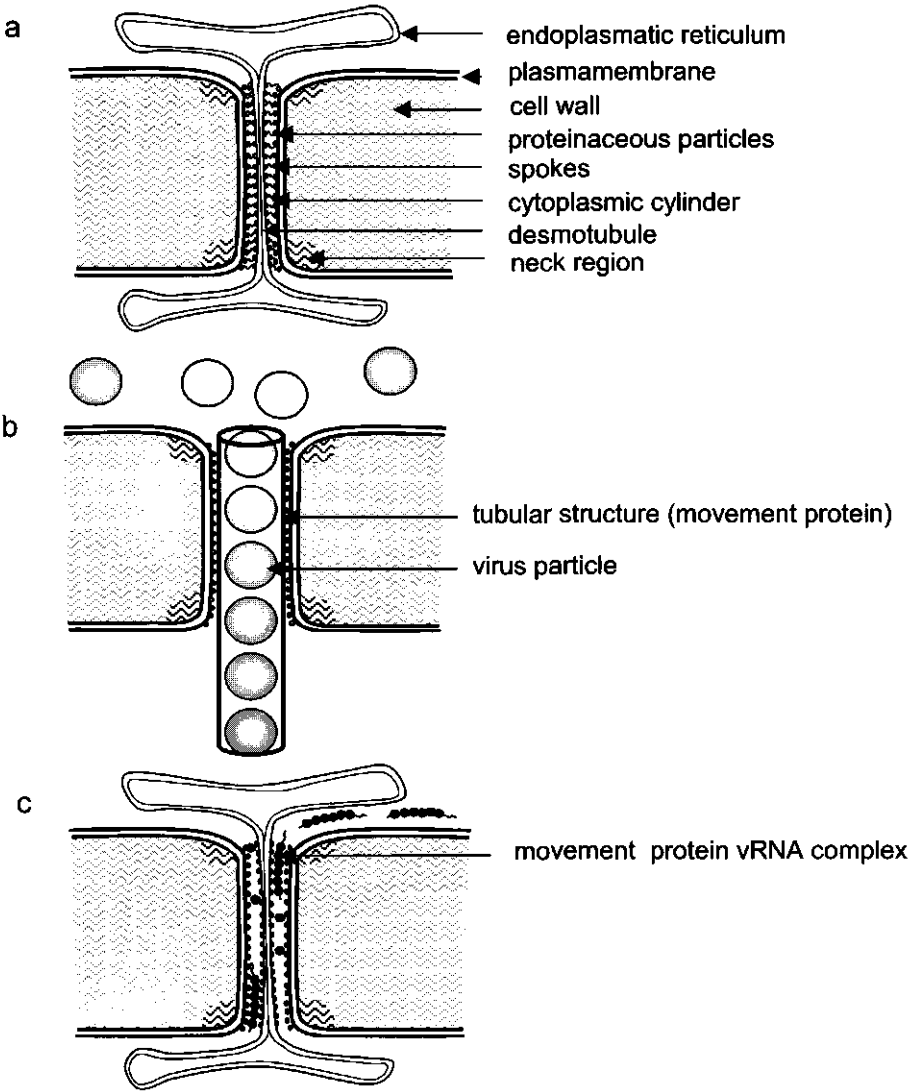
Figure 1.
Schematic model of a plasmodesma (a) and the modifications herein occurring during viral movement (b, c).
a) The endoplasmatic reticulum constructs a desmotubule, penetrating the plasmodesmatal pore and embedding proteinaceous particles. The desmotubule is interconnected via the spokes or bridging proteins with the continuous plasmamembrane and the particles herein.
b) Tubular structure of movement protein inserted in the plasmodesmatal pore, transporting virions from cell to cell. The desmotubule is absent.
c) Transport of complexes of movement protein and viral RNA (vRNA). The plasmodesmatal structure is preserved but gating properties have changed so that MP-vRNA complexes can pass.

separation is not complete. Channels through the cell wall, the plasmodesmata, provide a cytoplasmic continuum. In these plasmodesmata the plasma membrane is continuous and an endoplasmatic reticulum (ER) cylinder is appressed into a desmotubule (Figure 1a). The desmotubule plays a potential role in the exchange of lipophilic signals, while ions and small molecules are transported through the cytoplasmic cylinder between the plasma membrane and the desmotubule (reviewed by van der Schoot and Rinne, 1999). This cylinder is thought to be subdivided into subchannels by the presence of so-called spokes that connect the desmotubule to the peripheral plasma membrane cylinder (Figure 1a). Based on ultra-structural data, it has been suggested that molecules of approximately 1 kDa can freely pass through the plasmodesmal pore (Overall *et al.*, 1982). Micro-injection studies using fluorescently labelled probes indeed confirmed that plasmodesmata have a size exclusion limit (SEL) of approximately 1 kDa, i.e. only molecules of 1 kDa and smaller diffuse through them from cell to cell (Goodwin, 1983). However, recent studies from Oparka *et al.* (1999) demonstrate that the SEL property can vary depending on the developmental stage of the leaf. In sink leaves, the metabolite importing leaves, plasmodesmata allow diffusion of macromolecules up to 50 kDa. During transition from sink to source (metabolite exporting leaves), the ultrastructure of the plasmodesmata appears to change from linear to branched (see paragraph on plasmodesmata formation) and their capacity to pass on macromolecules by diffusion is lost. Apparently, diffusional transport becomes severely restricted as soon as tissues mature.

How the plasmodesmal SEL is regulated or how the mechanism of transport through plasmodesmata is facilitated is not yet fully understood but some aspects have been elucidated. Cytoskeleton elements appear to be involved in the transport regulation as both actin and myosin elements have been localised to plasmodesmata (Blackman and Overall, 1998; Radford and White, 1998; White *et al.*, 1994). De-polymerisation of actin resulted in widening of the plasmodesmal pore and an increase in SEL (White *et al.*, 1994; Ding *et al.*, 1996). Therefore, the spokes connecting the proteinaceous particles in the plasma membrane to the desmotubule (Figure 1a) have been suggested to be composed of myosin elements (Overall and Blackman, 1996). Considering its biochemical properties, myosin could function as the active motor protein, facilitating transport through the plasmodesma.

In addition phosphorylation processes are thought to play a role in plasmodesmal gating. Treatments like application of azide or anaerobic conditions, which decreases cellular ATP levels, enlarge the plasmodesmal SEL (Tucker, 1993; Cleland *et al.*, 1994). In contrast, second messengers including Ca^{2+} and inositol phosphates, which activate protein kinases, reduce the plasmodesma SEL (Erwee and Goodwin, 1983; Tucker, 1988; 1990; Tucker and Boss, 1996). Recently, a calcium dependent protein kinase has been detected in isolated plasmodesmata fractions (Yahalom *et al.*, 1998).

A rather different mechanism to regulate plasmodesmata conductivity is effectuated by the deposition of callose in the neck region (Figure 1a) (Olesen and Robards, 1990). It is likely that various mechanisms for SEL regulation might serve specific processes or responses of the plant (Rinne and Van der Schoot, 1998).

In the last few years, a number of plasmodesmal components have been identified. Besides the already mentioned calcium dependent protein kinase (Yahalom *et al.*, 1998), Epel *et al.* (1996) isolated a 41 kDa protein from maize mesocotyl cell walls which is localised to plasmodesmata. In a similar approach with nodal walls of the green alga *Chara corallina* a 45 kDa plasmodesmal protein was isolated (Blackman *et al.*, 1998). Waigmann *et al.* (1997) isolated proteins from maize cell walls and generated two monoclonal antibodies (JIM64 and JIM67) that recognised putative plasmodesmal compounds in *N. clevelandii*. In regenerating protoplasts, outer-wall plasmodesmata which are destined to disappear again, show immuno-labelling using antiserum against ubiquitin (Ehlers *et al.*, 1996). It is speculated that ubiquitin may function in the decline of plasmodesmata on the protoplasts. The function of the other above mentioned putative plasmodesmata compounds still is largely unknown, and is subject of further research.

Plasmodesmata formation

In young tissue plasmodesmata arise during cell division in the forming cell plate due to entrapment of tubular strands of ER. These cytokinetically formed plasmodesmata are generally referred to as primary plasmodesmata. Secondary plasmodesmata are formed post-cytokinetically through non-division cell walls under circumstances described later. This original classification of primary or secondary plasmodesmata is based on ontogeny. In some cases a different classification was used for example, in young and older tobacco leaves the terms primary and secondary were used to refer to linear and branched plasmodesmata (Ding *et al.*, 1992). In this thesis the terms primary and secondary (or *de novo*) plasmodesmata are used in their original definition thus referring to their ontogeny and the terms linear and branched plasmodesmata are used to describe their morphology.

Formation of primary plasmodesmata has been reviewed in 1976 by Jones but the formation of secondary plasmodesmata has received little attention. Observations in graft interfaces, however, led to a model in which the cell wall of neighbouring cells is synchronously thinned so that opposing ER strands can be interconnected and will form new desmotubules (Kollmann and Glockmann, 1991). The fact that plants have the capacity to induce secondary or novel plasmodesmata, linear or branched, in non-dividing walls has been described in several studies with grafts (Jeffree and Yeoman, 1983; Binding *et al.*, 1987) and has been recognised as a normal event during plant development, i.e. in shoot meristems (van der Schoot and Rinne 1999). Furthermore, novel plasmodesmata were described between host and parasite, in elongating cells, between fusing carpels, between epidermal and underlying cells, during the sink-source transition of veins, between fusing protoplasts and between pollen mother cells (Tainter, 1971; Jones, 1976; Dell *et al.*, 1982; Schnepf and Sych, 1983; Seagull, 1983; van der Schoot *et al.*, 1995; Volk *et al.*, 1996). However, in each of these examples, intermediate stages of the plasmodesmal formation have not been reported and therefore, the suggested mechanisms for plasmodesma formation have remained speculative.

Induction of plasmodesmata branching has been suggested during infection of two different plant viruses, namely *Tobacco mosaic virus* (TMV) (Ding *et al.*, 1992) and

Maize streak virus (Dickinson *et al.,* 1996). The suggestion is based on the localisation of the (putative) MP exclusively on branched plasmodesmata. The induction of novel plasmodesmata for the transport of viruses has been thought plausible (Lucas *et al.,* 1993), but so far remained a matter of speculation.

Viral movement through plasmodesmata
To establish systemic infection plant viruses need to spread from the initial infection site throughout the whole plant. Movement of the virus proceeds in two phases namely the spread from cell to cell, within the parenchyma cell layers and transport over long distances via the vascular system (reviewed in Carrington *et al.,* 1996) . For movement from cell to cell viruses exploit plasmodesmata. Since the normal functional size of plasmodesmata does not permit their passage, plant viruses require a mechanism to modify these channels to enable the transport of their genome or particle. For this purpose plant viruses encode one or more movement proteins (MPs).

Using temperature-sensitive mutants the MP of TMV was first identified, as a 30 kDa protein (Leonard and Zaitlin, 1982). This MP was later localised to plasmodesmata of infected leaves (Tomenius *et al.,* 1987) and since then, MPs have been identified for numerous viruses and an increasing number of the viral MPs has been immuno-localised to plasmodesmata (for reviews see: Hull, 1989; Maule, 1991; and Carrington *et al.,* 1996). Sequences similarities between the MP genes of a wide range of viruses revealed the existence of conserved motifs (Melcher, 1990; Koonin *et al.,* 1991) although, the proteins appear to function in different manners.

So far, two distinct mechanisms for intercellular virus movement have been described, namely the movement of whole virions and the movement of viral genomes in non-virion form (reviewed in Deom *et al.,* 1992; Carrington *et al.,* 1996). A conspicuous feature of the first movement mechanism is the tubular structure, which is assembled by MP copies and inserted in the plasmodesmal pore to transport virions (Figure 1b). The tubule-guided movement mechanism is exemplified by the comoviruses and has been reported for an increasing number of plant viruses (reviewed in PhD thesis Kasteel, 1999). Obviously, the coat protein (CP) is required for this type of movement, a distinctive difference with the second mechanism in which the genome is transported in a non-virion form.

The CP can be abolished during the latter type of movement, which is exemplified by TMV (Dawson *et al.,* 1988). The TMV MP is capable of binding ssRNA and ssDNA in a co-operative, non-specific manner with a minimal binding site of four to seven nucleotides per MP monomer (Citovsky *et al.,* 1990; 1992). The MP-RNA complex localises to plasmodesmata where the MP then modifies the plasmodesmata functionally, allowing transport of the viral RNA (Figure 1c). Experimentally, this modification is also visible as an increase in the plasmodesmal SEL and is prominent at the expanding infection site and in transgenic plants constitutively expressing the MP gene (Oparka *et al.,* 1997; Wolf *et al.,* 1989). In case of TMV, it is hypothesised that MP-RNA complexes are stretched out into thin structures that match the cytoplasmic subchannels of the modified plasmodesmata (Citovsky and Zambryski, 1991).

In addition, to the above mentioned mechanisms for virus movement, other mechanisms apparently must exist since, for example the movement of potyviruses, potexviruses and geminiviruses can not be explained in either way (reviewed in Storms, 1998). Tubular structures containing virions have never been found for these viruses, although they require their CP for cell-cell movement.

Alfalfa mosaic virus

Alfalfa mosaic virus (AMV) was first described in 1931 by Weimer as the agent causing mosaic symptoms in lucerne (*Medicago sativa* L.). AMV has a broad host range, able to infect over 305 species in 47 dicotyledonous families (Hull, 1969). The virus causes a variety of symptoms in different plant species, ranging from mosaic in lucerne, tobacco, clover and chilli pepper, calico and tuber necrosis in potato, calico or mosaic in celery and lettuce, severe necrosis in tomato, necrotic streak in pea and necrotic local lesions in cowpea and broad bean. The virus occurs naturally in many other herbaceous and some woody species. The symptoms of AMV greatly depend on the host variety but the severity of the symptoms is also influenced by the virus strain and the environmental conditions. The geographical distribution of AMV is world-wide and the virus is transmitted by aphids or mechanically.
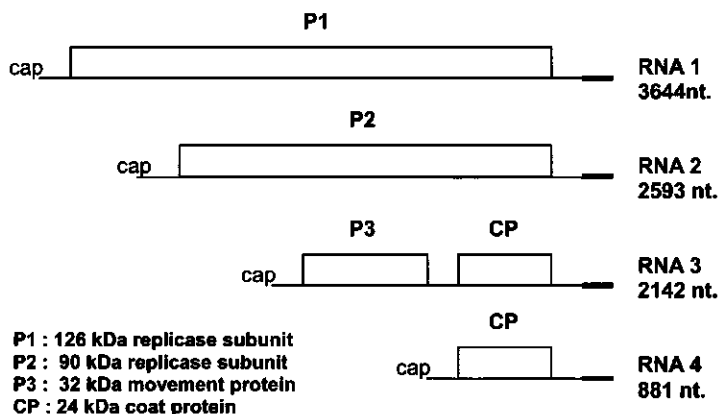

Figure 2. *Schematic representation of the genomic organisation of AMV. The genome consists of three genomic RNAs 1,2,3 and one subgenomic RNA 4.*

AMV is member of the *Bromoviridae* family which is subdivided into four genera: the bromoviruses, the cucumoviruses, the ilarviruses and the alfamoviruses with type species AMV. All *Bromoviridae* have a single stranded tripartite RNA genome of plus stranded polarity. The genome of AMV (Figure 2) consists of three genomic RNAs and a subgenomic RNA 4, which is derived from the RNA 3 negative template by recognition of a subgenomic promoter in the intergenic region. All RNAs have a cap structure and a homologous 3'-terminus region of 145 nucleotides (Figure 2). RNAs 1 and 2 are monocistronic and encode 126 kDa and 90 kDa proteins, referred to as P1 and P2 respectively. These proteins are two subunits of the viral RNA dependent RNA polymerase (RdRp) (Quadt *et al.*, 1991). RNA 3 is dicistronic and encodes the 32 kDa MP and via RNA 4, the 24 kDa CP. The MP and the CP have numerous functions during AMV infection as will be discussed separately hereunder.

AMV coat protein
A distinct function of the CP is the encapsidation of RNA into virions. A single copy of RNA 1, 2, 3 or two subgenomic CP messenger RNAs are encapsidated in bacilliform and icosahedral virus particles with a constant diameter of 19 nm and lengths ranging from 58, 48, 36 to 19 nm respectively (Figure 3). The different virus particles are, in decreasing sedimentation velocity, referred to as components B (bottom), M (middle), Tb (top) and Ta. Besides the four major AMV particles, at least 13 minor bacilliform components have been detected in purified virus preparations (Bol and Kaashoek, 1974).
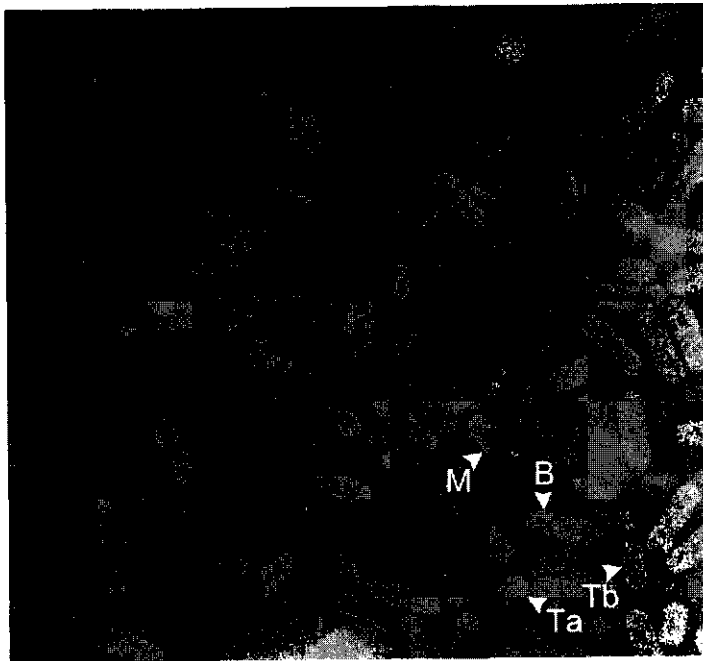
Figure 3. *Electron micrograph of bacilliform and icosahedral AMV virus particles. The virus particles are designated B, M, Ta and Tb. From Brunt et al., 1996*

For the four major particles, the number of CP monomers is adapted to the length of the RNA. Based on the particle weight, the RNA weight fractions and the molecular weight of the CP, Heijtink *et al.* (1977) calculated the number of CP monomers in the AMV particles is $60 + (n \times 18)$, n being the number of rings of 18 subunits (n = 10, 7, 5 or 4) which is inserted between rounded ends of a 60-subunit.

The monomers of the CP consists of a rigid core and a flexible N-terminal part of 36 amino acids (a.a.) residues (Kan *et al.*, 1982). In the virus particle, this part of the CP is immobilized, although there still is internal mobility (Andree *et al.*, 1981). The CP can be divided into a hydrophilic outer part and a hydrophobic inner part. The latter stabilises the interaction between two CP molecules, which results in the formation of dimers. The dimers behave water soluble and are the units from which the virion shell is constructed. Under the right conditions, the protein dimers can assemble into empty T=1 icosahedral particles (Driedonks *et al.*, 1977) which can be crystallised after the removal of the first 26 residues of the highly basic N-terminal part of the CP (Fukuyama *et al.*, 1983; Kumar *et al.*, 1997). X-ray diffraction data of these icosahedral particles demonstrated at a 4.0 Å resolution the presence of wide holes at the icosahedral. Reconstruction of the AMV T=1 particle with cryo-electron microscopy confirms the X-ray models (Kumar *et al.*, 1997) and also demonstrates the presence of large holes in the particles, which makes the virions accessible for spherical particles with a radius of greater than 17Å. Modelling experiments and negative staining electron microscopy, suggest that the naturally occurring bacilliform particle might have similar openings, at least as large as those in the T=1 particles (Hull *et al.*, 1969; Mellema, 1975). This would explain the susceptibility of the virions to RNase *in vitro* (Bol and Veldstra, 1969). Furthermore, the porous virions are unstable at high ionic strength and in the presence of sodium dodecyl sulfate suggesting that AMV virions are stabilised by RNA-protein interactions rather than protein-protein interactions (Kaper 1973).

Interaction of CP with viral RNA is crucial during AMV infection. A mixture of the three genomic RNAs is only infectious upon addition of a few CP molecules or the subgenomic messenger of CP (Bol *et al.*, 1971). This early function of the CP, called genome activation, is a process which is not yet fully understood. Possible roles of the CP in genome activation include: stabilising the viral genomic RNAs (Neeleman *et al.*, 1993); formation of an active replicase complex with other AMV proteins and putative host proteins (Quadt *et al.*, 1991); and later in the infection, inducing a switch from virus minus to plus-strand RNA synthesis (van der Kuyl *et al.*, 1991; van der Vossen *et al.*, 1994; and de Graaf *et al.*, 1995). Although lacking known RNA binding motifs, the N-terminal part of the CP is essential for RNA binding (Baer *et al.*, 1994; Yusibov and Loesch-Fries, 1995; 1998).

Besides the early functions, the CP is also involved the movement from cell to cell (Van der Kuyl *et al.*, 1991; van der Vossen *et al.*, 1994,1995). Based on two CP mutants CPN199 and CP4P it was concluded that the CP is essential for efficient cell-cell movement. Mutant CPN199 lacks 21 a.a. of the C-terminus of the CP (replaced by four non-viral a.a.) and mutant CP4P has an insertion of six non-viral a.a. at position 85. In protoplasts of transgenic P12 plants, which constitutively express the P1 and P2 proteins, both mutants still start genome activation and replicate the viral RNAs on a

reduced level. Also, CP can be detected at wild type levels but virions were not found. In P12 plants infection with mutant CP4P is not detectable and apparently the mutant can not move from cell to cell. For mutant CPN199 some CP is detected in inoculated leaves although at a 20-fold reduced level (Van der Kuyl *et al.*, 1991; van der Vossen *et al.*, 1994;1995). The fact that the RNAs of mutant CPN199, which are not encapsidated, can be isolated from the inoculated plants indicates that the mutant can move from cell to cell as non-encapsidated RNA or as a CP/RNA complex that does not survive the virus isolation. To be able to distinguish between these two options and thus determine the mechanism of AMV cell-cell movement, further research is needed.

AMV movement protein
The MP of AMV is a 32 kDa protein encoded by RNA3 (Figure 2). The protein accumulates at the beginning of the infection period and than disappears, so it is predominantly present during the initial stages of virus infection (Berna *et al.*, 1986). This is a property also described for MPs of other viruses, like TMV and *Tomato spotted wilt virus* (TSWV) (Lehto *et al.*, 1990, Kormelink *et al.*, 1994). In general, viral MPs are thought to be active in the plasmodesmata of plant cells, however, the AMV MP was detected in the middle lamella of walls surrounding cells at the expanding infection sites and only occasionally in the vicinity of plasmodesmata (Stussi-Garaud *et al.*, 1987; Godefroy-Colburn *et al.*, 1990). The protein was no longer detected in tissue, in which the virus had accumulated to high levels. In subcellular fractions of *Nicotiana tabacum* plants, AMV-MP was detected in the cell wall fraction of infected tissues and in the membrane fraction during the first stages of infection (Godefroy-Colburn *et al.*, 1986).

Likewise, the protein was found to be associated with cell walls of transgenic plants, expressing the MP gene (Erny *et al.*, 1992). Transgenic plants expressing a truncated form of the MP, lacking 12 a.a. from N-terminal part of the P3 sequence (P3 Δ[1-12]), behaved similarly. However a deletion of 77 a.a. from the N-terminus (P3 Δ[1-77]) abolished the cell wall targeting and the MP in these transgenic plants was detected only in the cytoplasm. So the N-terminal region of the AMV-MP contains the cell wall targeting domain. This region includes a negatively charged sequence, between a.a. 23 and 34, which probably forms a α-helix. Berna (1995) used mutant MPs with a deletion, an insertion or a substitution within a.a. 21-34, to determine whether the putative α-helix motif was essential for an optimal cell wall localisation, although sequences around the α-helix are also required in this process. For translocation to the plasma membrane the hydrophobic domain of the N-terminus (a.a. 46-67) could be involved.

In transgenic *N. tabacum* cv. Xanthi and cv. Xanthi *NN* plants expressing the truncated MP gene (P3 Δ[21-34]) confirmed the cytoplasmic localisation of the MP by immunogold labelling (Rohnfrisch *et al.*, 1996). These plants were also used for micro-injection studies of fluorescent probes to determine the SEL of plasmodesmata. The gating of the plasmodesmata in transgenic plants expressing both the complete MP gene and the truncated MPs, increased to a value between 4.4 kDa and 10 kDa, even though the MP could not be detected in plasmodesmata (Poirson *et al.*, 1993).

This increase in SEL, is less dramatic than the increase in SEL observed in transgenic plants expressing the TMV MP gene (Citovsky *et al.,* 1990). Nevertheless, the observation could be indicative for an AMV movement mechanism comparable with that of TMV. The capacity of the MP of AMV to bind single stranded nucleic acids also argues for this hypothesis. Schoumacher *et al.* (1994) determined that the RNA binding domain of the MP is included within a.a. 36-81, a region of the protein with a part of the positively charged domain (a.a. 69-90). The positive charge and a high probability of surface exposure are properties also described for the RNA binding domains of MPs of *Brome mosaic virus* (BMV), *Cauliflower mosaic virus* (CaMV), *Cucumber mosaic virus* (CMV), TMV, *Red clover necrotic mosaic virus* (RCNMV) (Fuijta *et al.,* 1998; Jansen *et al.,* 1998; Thomas and Maule, 1995; Vaquero *et al.,* 1997; Citovsky *et al.,* 1992; Osman *et al.,* 1993). Unlike the CP-RNA binding, the MP-RNA binding is not sequence specific.

The mechanism of AMV movement
Based on the data summarised above, a decisive perception of the mechanism of cell-cell movement can not be given. Properties of the MP as RNA binding, increasing the SEL and sequence homology with TMV, suggest a movement mechanism as described for the tobamo-viruses thus the transport of the RNA-MP complex. However, the CP of AMV is needed for cell-to-cell movement, a requirement which does not fit in the concept of this movement mechanism. The tubule guided movement mechanism might be employed by AMV although tubular structures induced by AMV were, at the onset of this research project, not described. Therefore, the first objective in this project was to elucidate the movement mechanism AMV exploits.

Scope and outline

The research described in this thesis was part of a larger programme entitled '*The role of plasmodesmata in virus transport and cell-cell communication*'. Within the framework of this programme three PhD research projects were simultaneously executed, aiming different, but complementary goals, and which as a whole would provide a better insight in the functioning of plasmodesmata. The aim of one project, executed at the Institute of Molecular Plant Sciences, Leiden University (PhD student L. Jongejan, supervisor J. Bol), was to identify host plant (*N. benthamiana*) proteins which interact with the movement and coat proteins of the two distinct viruses, AMV and CPMV, by two-hybrid analysis, as to identify candidate plasmodesmal proteins. The aim of the second project, executed at the Laboratory of Molecular Biology of the Wageningen University (PhD candidate P. Bertens, supervisor J. Wellink) was to characterise the functional domains within the MP of CPMV. The third project, of which the results are described in this thesis, deals with the *in situ* analyses of the movement proteins of AMV and CPMV in their interactions with host proteins and plasmodesmata.

At the onset of the project the movement mechanisms of AMV and CPMV were thought to be different, AMV using the tobamo-like movement mechanism and CPMV using the tubule guided movement mechanism. Surprisingly, in a single cell system, it

was established that the AMV MP, similar to CPMV had the ability to form tubular structures protruding from the surface of infected protoplasts (**Chapter 2**). These tubules were engulfed by plasma membrane and contained the AMV characteristic bacilliform and spherical virus particles. Using immunogold analysis the presence of both MP and CP was confirmed.

From previous studies using mutagenesis of the CP and MP gene, several AMV mutants were obtained which were blocked in their movement from cell-to-cell (CPN199, CP4P, SP6 and SP7; van der Vossen *et al.*, 1994;1995). These mutants have been studied (**Chapter 3**) for their potential to produce (empty) transport tubules on infected protoplasts, as to gain further evidence for a tubule-guided movement mechanism for AMV and moreover, to rule out or collect evidence for alternative movement mechanisms used by AMV.

To address the question whether AMV induces tubular transport structures in tissue, immuno-gold labelling was performed which localised both CP and MP to plasmodesmata at the infection border (**Chapter 4**). These plasmodesmata were structurally modified since the diameter of the plasmodesmal pore had increased, the desmotubule was absent and a chain of virions was occasionally detected. Therefore, the insertion of a tubular structure to transport virions was proposed.

In addition to the modification of the plasmodesmata, in the zone where active cell-cell movement of AMV occurs, the number of plasmodesmata was notably higher than in uninfected tissue and fully infected tissue (**Chapter 5**). From these results it was concluded that during cell-cell movement of AMV novel plasmodesmata were being induced. Also it was determined that the modified plasmodesmata still contained plasmodesmal compounds and therefore resembled plasmodesmata both structurally and biochemically.

Since AMV was capable of modifying pre-existing plasmodesmata and presumably induced novel plasmodesmata, it seemed likely that the viral MP interacts with host proteins to facilitate these alterations. The yeast two hybrid system was used to identify host proteins interacting with the MPs of AMV and CPMV (see thesis of L. Jongejan). The most promising candidate appeared to be the *ad3* gene, selected using the AMV MP as bait. Antiserum was raised against protein AD3 and used for *in situ* localisation studies (**Chapter 6**). These demonstrated that the AD3 protein is present in the subcellular membrane fraction of both root and leaf material of *N. benthamiana*. Immuno-gold labelling in leaf tissue revealed the AD3 to be localised at the plasma membrane although no co-localization with the AMV MP was detected

In **Chapter 7** the results from the previous chapters are discussed in view of the literature and based on these discussions, a model for the movement mechanism of *Alfalfa mosaic virus* is proposed.

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Chapter 2

Tubule forming capacity of the movement protein of *Alfalfa mosaic virus*

Summary

The structural phenotype of the movement protein (MP) of *Alfalfa mosaic virus* (AMV), a representative of the *Bromoviridae*, was studied in protoplasts. Immunofluorescence microscopy showed that the MP of the virus assembled into long tubular structures at the surface of infected protoplasts. Electron microscopy and immunogold analysis confirmed the presence of both MP, coat protein and virus particles in the tubules induced by AMV. The significance of the tubule-forming properties of the viral MP is discussed.

Introduction

Plasmodesmata, the intercellular channels of plant cells, are utilised by plant viruses to move particles or the viral genome from cell to cell. However, plasmodesmata only allow passive passage of molecules smaller than 1 kDa, in other words, the size exclusion limit (SEL) of plasmodesmata is < 1 kDa (Wolf *et al.,* 1989). Therefore, viruses are forced to modify the intercellular channels. For a growing number of viruses it has been shown that one or more virus-encoded proteins, referred to as movement proteins (MPs), are actively involved in this process. Among different viruses, the functioning of these MP varies, like the role of the coat protein (CP) varies during cell-to-cell movement.

One category of plant viruses, including *Tobacco mosaic virus* (TMV), does not require CP and moves in a non-virion form through plasmodesmata. The virus-encoded MP is localised to the plasmodesmata (Tomenius *et al.,* 1987) and causes a significant increase in the SEL. In addition, it has been reported that the viral MP is capable of binding ssRNA and based on these observations, it is suggested that a complex of viral RNA and MP is transported through the plasmodesmata (Wolf *et al.,* 1989). Another category of viruses, including *Cowpea mosaic virus* (CPMV), requires CP for intercellular spread and these viruses move as virions through tubules constructed of viral MP, which are assembled within the plasmodesmata (van Lent *et al.,* 1990). These movement tubules are not only formed in plant tissue but also on the surface of virus-infected protoplasts in the absence of cell walls (van Lent *et al.,* 1991). A third category of plant viruses, including *Tobacco etch potyvirus* (Dolja *et al.,* 1994) and *Potato virus X* (Oparka *et al.,* 1996; Santa Cruz *et al.,* 1998), also requires the CP for cell-to-cell movement but tubule-guided virion transport through plasmodesmata has not been reported.

The mechanism of cell-to-cell movement used by *Alfalfa mosaic virus* (AMV) still has to be elucidated. Both the MP and CP of AMV appear to be involved in this process of movement (Stussi-Garaud *et al.,* 1987; Dore *et al.,* 1991; van der Kuyl *et al.,* 1991; van der Vossen *et al.,* 1995). The AMV MP is capable of binding viral RNA (Schoumacher *et al.,* 1994) and transgenic plants expressing the AMV-MP demonstrate an increase in plasmodesmal SEL (Poirson *et al.,* 1993) similar to properties which have been described for TMV-MP. Targeting to the cell wall and transient presence are further features of the AMV MP (Stussi-Garaud *et al.,* 1987; Godefroy-Colburn *et al.,* 1986).

The CP of AMV has several functions during the infection cycle of the virus. An early function is the requirement of a few CP molecules for genome activation (Bol *et al.,* 1971, Jaspars *et al.,* 1985). Furthermore, CP has been detected in association with the RdRp complex which also contains the replicase proteins P1 and P2 and several unidentified host components (Quadt *et al.,* 1991). Based on mutagenesis, van der Kuyl *et al.* (1991) and van der Vossen *et al.* (1994) demonstrated the requirement of the CP during cell-to-cell movement of the virus. Although involvement of the AMV CP was established using these CP mutants, it was not possible to conclude whether AMV moves as a virion or in form of another type of CP-RNA complex. AMV mutant CPN199, with a C-terminal deletion in the CP, showed reduced spread in

leaves despite the fact that stable virions were not detectable (van der Vossen *et al.*, 1994).

Based on these observations, the movement mechanism of AMV can not be established yet. *In situ* observation and localisation of both proteins might shed more light on the form in which AMV is moved and the function of the MP and CP in this process. By analysing the phenotype of the MP in infected protoplasts, we have investigated whether AMV moves as whole virions through tubule-like structures.

Methods

Infection and analysis of protoplasts

Cowpea protoplasts (*Vigna unguiculata* 'California Blackeye') and protoplasts from *Nicotiana benthamiana* and *Nicotiana tabacum* Samsun NN were mock-inoculated with water or inoculated with AMV (strain 425) at a concentration of 10 μg virus per 10^6 protoplasts, essentially as described by Eggen *et al.* (1989). Forty-two hrs after inoculation, the protoplasts were analysed by negative staining electron microscopy and immunofluorescence (van Lent *et al.*, 1991) using antibodies against the MP or CP. Controls consisted of the mock-inoculated protoplasts and samples treated without the primary antisera. Samples were analysed using a Leitz Laborlux S fluorescence microscope and a Philips CM12 electron microscope.

Antisera

Antibodies against the CP of AMV were generated by injecting purified virus into rabbits and antibodies against the MP (P3 protein) were generated as described by van Pelt-Heerschap *et al.* (1987).

Results and Discussion

At 42 hrs post-inoculation, an average of 60% of the inoculated protoplasts was infected with AMV. Approximately 75% of the infected protoplasts showed numerous fluorescent tubular structures at the cell surface upon immunostaining with anti-MP serum (Figure 1a). These tubules were also visible, though to a lesser extent, when anti-CP serum was used, indicating the presence of CP in the tubular structures (Figure 1b). Tubules were also observed on AMV-infected protoplasts from *N. benthamiana* and *N. tabacum* Samsun NN (data not shown).

Electron microscopical examination of whole mounts of AMV-infected protoplasts was performed by negative staining with 2% (w/v) phosphotungstic acid (PTA) at either pH 5.5 or 6.5. These two pH values were chosen as AMV particles are stable at pH 5.5, but considerably less stable at pH 6.5. In preparations stained with PTA at pH 5.5, virus-like particles were observed within the tubules (Figure 2a,b). However at pH 6.5 no such particles could be visualised within the tubules or in the background (Figure 2c). Repeatedly, several tubular structures engulfed by plasma membrane were observed (Figure 2). The phenomenon of multiple tubules enclosed by plasma membrane was also reported for CPMV (van Lent *et al.*, 1991). The average diameter
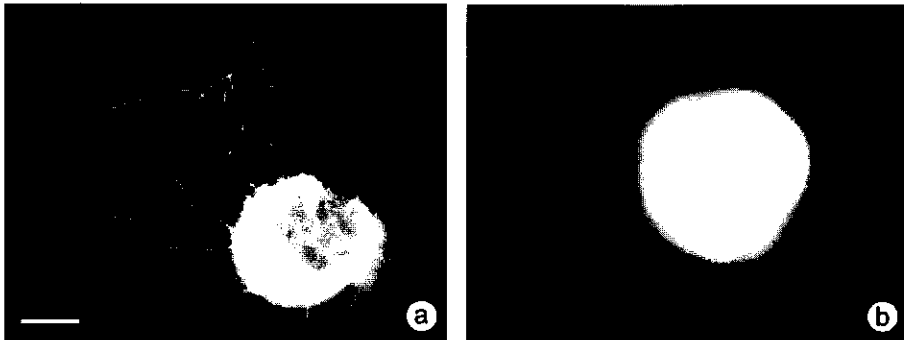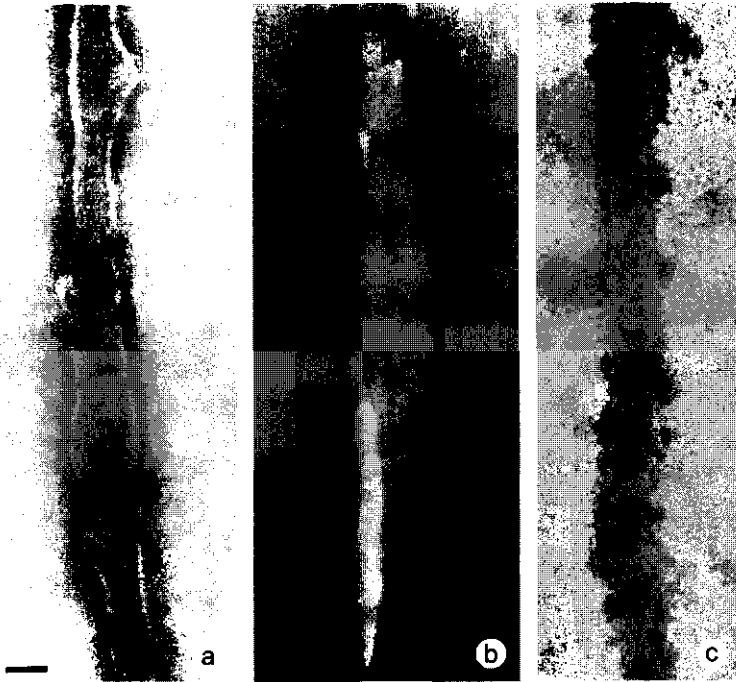
Figure 1
Immunofluorescent images of cowpea protoplasts 42 hrs after inoculation with AMV, labelled with anti-MP serum (a) or anti-CP serum (b). Bar represents 10 µm.

of the tubules was 25 ± 3 nm. The particle-like structures observed in the tubules (Figure 2b,c) had a diameter of 17 nm, similar to the diameter reported for AMV. The correct diameter, the spherical and bacilliform structures as well as the instability of the particles at higher pH confirm that the particulate structures observed within the tubules are indeed mature AMV virions.
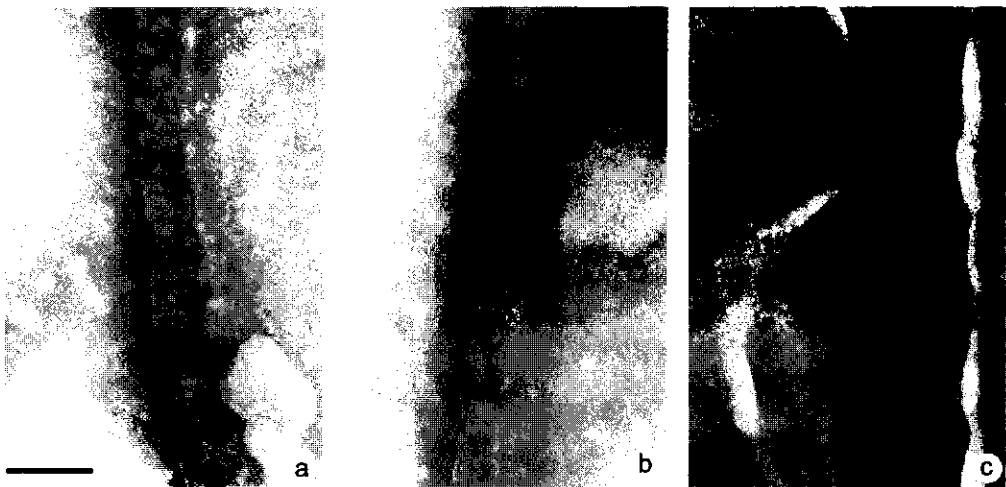
Figure 2
Electron micrographs of tubular structures of AMV, negatively stained with PTA at pH 5.5 (a,b) or pH 6.5 (c). Icosahedral and bacilliform virions are visible within the tubular structure at pH 5.5. Tubular structures are engulfed by plasma membrane. Bar represents 100 nm.

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

The presence of AMV CP in the tubules was confirmed by immunogold labelling with anti-CP serum. Gold label was found on the tubules, in particular at sites where the structure had partly disintegrated (Figure 3a). No gold labelling was found on intact AMV tubules, but gold complexes were found at places where virus particles were freely accessible e.g. at the end of a tubule (Figure 3b). Apparently, the immunoglobulins can not reach the CP antigen when tubules are intact, as has been found for CPMV tubules (van Lent *et al.*, 1991).

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

It is evident that AMV can induce tubular structures in infected protoplasts and that these tubules contain the MP and occlude virus particles. This complements the observations of van der Vossen *et al.* (1994) on the requirement for CP for intercellular movement of AMV. Hence, by analogy with CPMV intercellular

movement, it is plausible that movement of mature virions through tubules assembled in plasmodesmata is a valid mechanism for AMV movement in plant tissue. In this respect, Godefroy-Colburn *et al.* (1990) noted the transient presence of tubule-like structures, gold labelled with anti-CP, in plasmodesmata of AMV-infected tobacco mesophyll parenchyma cells. Also, for members of two other genera of the family *Bromoviridae*, tobacco streak ilarvirus (Martelli and Russo, 1985) and tomato aspermy cucumovirus (Francki *et al.,* 1985), tubular structures containing virus particles have been observed in plasmodesmata of infected plant cells. However, the presence of virus-containing tubular structures in plasmodesmata of AMV-infected plant tissue remains to be established.

Acknowledgements

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Chapter 3

Cytological analysis of movement defective *Alfalfa mosaic virus* mutants.

Summary

To gain further insight in the cell to cell movement mechanism of *Alfalfa mosaic virus*, four different mutants, deficient in this process, were cytologically analysed using immuno-fluorescence and electron microscopy. Two coat protein mutants, CP4P and CPN199, disturbed in the formation of stable virions were tested for their ability to form empty movement tubules on single protoplasts. Empty tubular structures were indeed detected for CP4P but not for CPN199. Two movement protein mutants, SP6 and SP7 did not induce tubular structures on infected protoplasts. The results indicate that AMV cell-to-cell movement depends on the virus ability to produce movement protein containing tubules and stable virions.

Introduction

Alfalfa mosaic virus is a tripartite RNA virus, the RNAs 1 and 2 encoding the replicase subunits P1 and P2 and RNA 3 both the movement protein (P3 or MP) and, via a subgenomic messenger, the coat protein (CP) (Figure 1). The CP binds *in vitro* to the 3'-terminal region of RNAs 1, 2 and 3 with a specific and high affinity (Houser-Scott *et al.*, 1994; 1997; Reusken *et al.*, 1994; 1996) and this interaction is thought to be essential for further phases in the infection cycle (review Bol, 1999).

An early function is the requirement of a few CP molecules for the initiation of the infection, a process referred to as genome activation (Bol *et al.*, 1971; Jaspars *et al.*, 1985). Binding of N-terminal CP peptides (25, 26 and 38 amino acids (aa)) to the 3' terminal 39 nucleotides of AMV RNAs was demonstrated as well as their ability to activate the genome (Bear *et al.*, 1994). In line with this, deletions in the N-terminus of the CP (aa 2-20) did abolish genome activation (Yusibov and Loesch-Fries, 1995; van der Vossen *et al.*, 1994). In transgenic plants expressing the replicase subunits P1 and P2 (P12 plants), the early function of the CP is no longer required (Taschner *et al.*, 1991). In addition, the CP has been detected in association with the replicase complex containing P1 and P2 and several unidentified host components (Quadt *et al.*, 1991). Isolation of this RNA-dependent RNA-polymerase can be done and results under the correct conditions in a functional complex (Quadt *et al.*, 1991).

Based on mutagenesis, van der Kuyl *et al.* (1991) and van der Vossen *et al.* (1994) demonstrated the requirement of the CP for cell-to-cell movement of the virus. However, it is unclear whether the CP needs to assemble into virions or functions here in a non-virion form. For a number of viruses assembly of the CP into virions has been shown to be required for cell-to-cell movement through tubular structures (van Lent *et al.*, 1990; Ritzenthaler *et al.*, 1995). The tubular structures produced by AMV on infected protoplasts (Kasteel *et al.*, 1997, Chapter 2) strongly suggest that AMV likewise uses a tubule guided movement mechanism and transports whole virions from cell to cell via these structures.

On the other hand, there is some evidence suggesting that the AMV CP might function in a non-virion form during cell-cell movement. Mutant CPN199, a CP mutant with a truncated CP gene encoding the 199 N-terminal amino acids (Figure 1), and for which no virions could be isolated from infected protoplasts, still was able to spread from cell to cell in plants, albeit at a low, reduced level (van der Vossen *et al.*, 1994). This suggests that AMV is also able to move in a non-virion form.

To investigate this apparent contradiction and, more general, to address the question whether the formation of virion-containing transport tubules is critical for successful AMV transport, in this chapter a series of available AMV mutants, all deficient in cell-to-cell movement, was studied. These mutants, of which the genetic make-up is depicted in Figure 1, include two CP mutants (CPN199 and CP4P) and two MP mutants (SP6 and SP7). Using P12 *N. tabacum* plants, which transgenically express the AMV replicase subunits, it was shown previously (van der Vossen *et al.*, 1994) that the CP mutant CP4P (containing a 4 aa insertion at position 85) is not able to move from cell to cell, but CP mutant CPN199 does. The expectation would therefore be that upon infection of single P12 protoplasts, the former mutant would produce
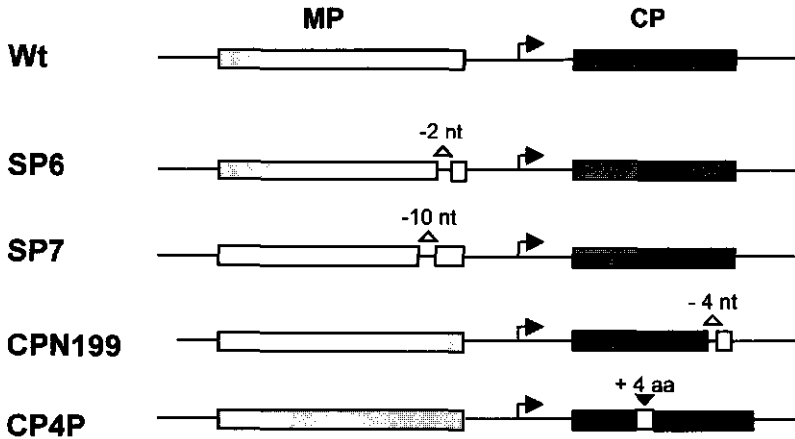
Figure 1
Schematic representation of genomic RNA3 of AMV wild type (Wt) and mutants CPN199, CP4P, SP6 and SP7 originally described by van der Vossen et al., 1994; 1995). Light and dark shaded boxes represent Wt open reading frames of the MP and CP respectively and white boxes indicate non-viral amino acids (aa). The arrow indicates the start position of the subgenomic promoter and the arrowheads pointing upwards indicate a deletion or, pointing downwards an insertion. A deletion of 2 respectively 10 nucleotides (nt) at the C-terminal end of the MP was made (mutants SP6 and SP7) and leads to a frame shift resulting in the lack of 3 respectively 10 aa at the C-terminus. The deletion of 4 nt from the CP gene causes the encoding of 199 N-terminal aa followed by 4 non-viral aa (mutant CPN199). CP mutant CP4P was made by the insertion of 4 extra aa at position 85.

empty transport tubules, whereas mutant CPN199 should produce at least some virion containing tubules on these cells.
Besides these two CP mutants also two MP mutants denoted SP6 and SP7 (Figure 1) were included in the protoplast infection studies, in an attempt to obtain further support for the hypothesis that AMV would move solely in virion-form through the MP containing transport tubules. Both mutants contain small, but different deletions in the C-terminus of the MP and have both been shown to be defective in systemic spread (van der Vossen *et al.*, 1995), using again the P12 plant infection system. As they are able to form mature virions, the expectation for these mutants is therefore that on infected protoplasts either empty or no tubules at all are to be found.

Methods

AMV CP and MP mutants
The MP mutants SP6, SP7 and CP mutants CPN199, CP4P were kindly provided by Prof. J.F. Bol. The mutations made in the AMV genome, are depicted in Figure 1 and comprehend two small deletions in the C-terminus of the MP (SP6 and SP7), a

deletion in the C-terminus of the CP (CPN199) and an insertion in the CP at position 85 (CP4P) (van der Vossen *et al.*, 1994;1995)

Inoculation of protoplasts

Protoplasts were prepared according to van Dun *et al.* (1988) from tobacco plants expressing the AMV P1 and P2 genes (P12 plants, also a gift of Prof. J.F. Bol). P12 plants were grown as described by Taschner *et al.* (1991). In summary, seeds were germinated on MS medium plates containing kanamycine and the antibiotic resistant seedlings were grown in plastic boxes containing MS medium. The protoplasts were isolated from young leaves and per sample 2.5 x10^5 protoplasts were inoculated with 30 µl of transcript (3 µg RNA) using electroporation. The protoplasts were incubated in constant illumination at 25°C for 16 hours.

Analysis infected protoplasts

Protoplasts were prepared for immuno-fluorescence and negative staining electron microscopy 0 h and 16 h post inoculation as described in Chapter 2 and by Kasteel *et al.* (1997). The antisera used were raised against the CP and a C-terminal peptide of the MP of AMV (van Pelt-Heerschap *et al.*, 1987) or antiserum raised against a 12 aa N-terminal deleted form of the AMV MP, a kind gift of Prof. C. Stussi-Garaud. Negative staining electron microscopy was done, using 2% phosphotungstic acid (PTA) pH 5.5 on infected protoplasts unfixed and fixed with 3% gluteraldehyde, 2% paraformaldehyde in phosphate-citrate buffer, pH 5.3 (0.1 M Na$_2$HPO$_4$ · 2H$_2$O, 2.7 mM citric acid). To trap virions from the cell suspension, immuno-sorbent electron microscopy (ISEM) was performed using CP antiserum to coat the grids.

Cytological analysis of infected plants

Plants were inoculated with wt-RNA and mutant SP7, CP4P and CPN199 RNA on two lower leaves of young 3-4 cm P12 plants according to Taschner *et al.* (1991). The inoculated plants were analysed 4 days and 7 days post inoculation (dpi) by light microscopic detection of the CP (after Christie and Edwardson 1986). Essentially, the leaves were sectioned in ribbons of approx. 1 mm, fixed in 3% glutaraldehyde, 2% paraformaldehyde in phosphate-citrate buffer, pH 5.3, rinsed in PC buffer and incubated in 5% (v/v) Triton-X-100 for 10 min. Subsequently, the ribbons were dehydrated in 30, 50, 70, 80, 96% ethanol, rinsed in water and PBS and blocked in 5 % (w/v) ELK in PBS for 1h. Incubation of the ribbons in the antiserum against the CP (1:1000) was done in PBS-Tween (0.05% v/v) with 10% healthy leaf extract for 2 h. The samples were thoroughly washed in PBS-Tween for 1 h and incubated in goat anti-rabbit antibodies conjugated to alkaline phosphatase (dilution 1:5000) in PBS-Tween for 1h and again washed in PBS for 0.5 h and AP buffer (pH 9.5) for 0.5 h. Finally, the CP was visualised using NBT, BCIP substrate until the purple substrate was visible using light microscopy.
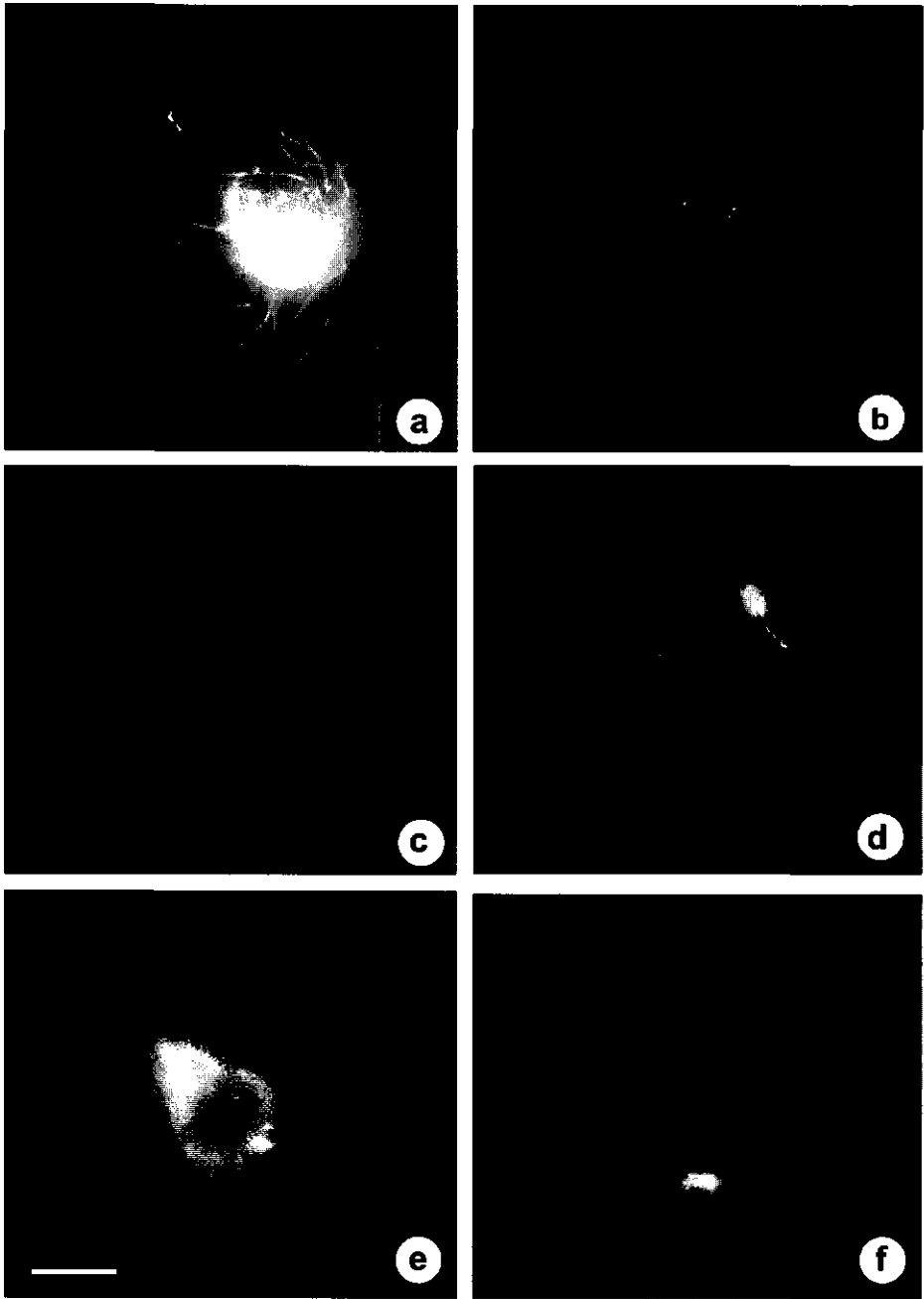
Figure 2
Presence of MP in P12 protoplasts, 16 h post infection with wt AMV (a,b) CP mutant CP4P (e,f) and CPN199 (d) and MP mutant SP7 (c) visualised by immuno-fluorescence using antiserum against the MP. Bar represents 10 µm

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Results and Discussion

Imaging the transport tubules of CP mutant CPN199 and CP4P

As both CP mutants CPN199 and CP4P encode wildtype MP, transfection of P12 protoplasts with either mutant was expected to result in tubule formation. To challenge the question if these tubules would contain virions, P12 *N. tabacum* protoplasts were transfected with wildtype or mutant *in vitro* RNA3 transcripts. Between different transfection experiments the percentage of infected protoplasts, both with mutant and wild type constructs, varied between 5 and 55%, demonstrating an apparent difficulty to reproducibly transfect P12 protoplasts with AMV RNA. Using antibodies against the CP, wildtype infected protoplasts always showed a brighter fluorescence than protoplasts infected by either mutant. This contradicts earlier conclusions by van der Vossen *et al.* (1994), who found comparable levels of CP for wildtype and mutants by Western blot analysis. Despite this discrepancy it could be concluded from the immunofluorescence data that both CPN199 and CP4P successfully multiplied in P12 protoplasts.

The immuno-fluorescent labelling of MP in AMV infected protoplasts demonstrated two main features; i.e. tubular structures (Figure 2 a,e) and intracellular, filamentous patterns (Figure 2 b,d,f). These latter patterns were detected 16 h after transfection, both with wild type and CP mutants. Probably due to lower replication levels, the number of tubules formed by mutant CPN199 was limited allowing detection by fluorescence microscopy but not by electron microscopy. Mutant CP4P did produce sufficient tubules on P12 protoplasts to allow further characterisation. Tubular structures present on CP4P transfected protoplasts, were analysed by negative staining electron microscopy and appeared to be empty, while the tubular structures of the wild type contained virions using the same protocols (Figure 3).

When determining if AMV employs a tubule guided transport mechanism, it is crucial to establish whether the CP mutants, defective in their cell-to-cell movement, could still produce virions. By electron microscopical analysis of the infected protoplasts, no virions could be detected for both CP mutants CP4P and CPN199 (Table 1), while in the same transfection experiment the wild type did produce virions. To exclude the possibility that unstable virions remain undetected using the standard staining procedures, extra fixation of the samples and coating of the grids with the CP antiserum (ISEM) was applied but did not result in detection of virus particles. These results are supportive for a tubule guided movement mechanism involving whole virions as the transport form.

Behaviour of MP mutants SP6 and SP7 in P12 protoplasts

To gain further support for the theory that successful cell-to-cell movement of AMV is unconditionally linked to the occurrence of virion-filled tubules, two MP mutants, blocked in their cell-to-cell movement were studied in P12 protoplasts. It has been demonstrated by van der Vossen *et al.* (1995) that both SP6 and SP7 replicate in P12 protoplasts but are defective in cell-to-cell movement in P12 plants. As both mutants produce wild type virions in single cells, it was expected that their multiplication in P12 cells would not result in tubule formation. Indeed, while protoplast transfections
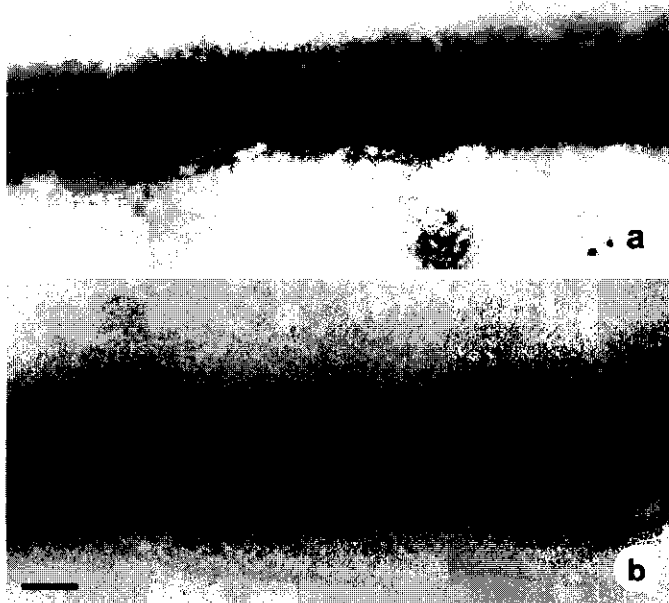
Figure 3
Electron micrographs of negatively stained tubular structures of wt AMV (a) and CP mutant CP4P (b). Bar represents 50 nm.

with wildtype AMV RNA3 resulted in numerous tubules, in different repetitions MP mutants SP6 and SP7 transfections never produced tubular structures in the same experiments using the same protoplast batches (Figure 2, Table 1). Although negative evidence, these data do not contradict the hypothesis of tubule guided movement as the sole cell-to-cell movement mechanism employed by AMV. Immuno-fluorescent images of SP6 or SP7 transfected protoplasts lacked the intracellular patterns detected for the wildtype and CP mutants (Figure 2). This may suggest that the mutants MPs are not stable which also would clarify the absence of tubular structures.

Table 1. *Summary of results AMV mutant infections in P12 protoplasts and plants. The accumulation of CP and MP and the presence of tubular structures were analysed using immuno-fluorescence and electron microscopy and is represented as ++++ wt level, +++ near wt, ++ reduced, + low levels, − not detected and n.d. not done. The accumulation of CP in inoculated leaves of P12 plants was analysed using light microscopy and is represented as wt levels (++++), reduced (++) or was not done (n.d.). * No virions detected in tubules **Accumulation in cortex tissue and in single epidermal cells.*

		WT	CP4P	CPN199	SP7	SP6
Accumulation in protoplasts	CP	++++	++	+	++++	++++
	MP aggregate	++++	+++	++	−	--
	MP tubules	++++	++	+	−	−
	Tubules in EM	++++	++*	−	−	n.d.
	Virions in EM	++++	−	−	+	n.d.
Accumulation CP in plants		++++	++**	++**	++**	n.d.

Limited cell to cell spread by AMV mutants is cell specific

It was previously reported that mutants CP4P and SP7 are defective in spread through P12 plants (van der Vossen *et al.*, 1994; 1995). Detailed analysis of various tissues revealed in different repetitions limited spread of these mutants and of CPN199. In parenchyma tissue, CP produced by mutants CP4P, CPN199 and SP7 could be detected merely in single and occasionally neighbouring epidermal cells (Figure 4b) and in some causes single leaf hair cells. Clearly, both the MP mutant and CP mutants could not move from cell to cell in parenchyma tissue. In contrast, the wild type virus dispersed through parts of the leaf within 4 days and infected large parts of the leaf within 7 days, although not all cells were infected (Figure 4a,c).
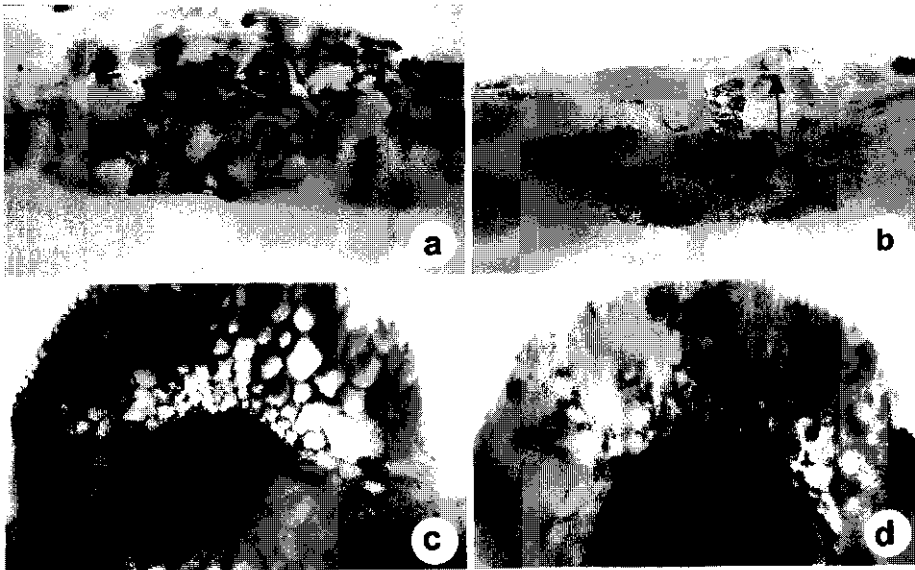
Figure 4
Light microscopical detection of viral CP in cross sections of P12 leaves inoculated with wt AMV (a, c) or CPN199 (b, d) 4 days post inoculation. Arrow indicates leaf cell infected with CPN199 mutant virus (b). Sections were made through leaf parenchyma tissue (a, b) or veins (c, d).

Surprisingly, patches of CP containing cells were detected in the cortex cells, surrounding the veins (Figure 4d) from leaves inoculated with either CP or MP mutants. This was an unexpected but reproducible observation. So despite the fact that the mutants lack either stable virions (CP mutants) or tubular structures (MP mutant), two components essential for movement in parenchyma cells, these mutants still translocate locally in cortex tissue.

An explanation for this phenomenon could be in the difference in cytoplasmic conditions between the cell types. A factor like the pH influences the stability of proteins and mutant CP or MP may be more stable in cortex cells than in parenchyma cells. Further research is needed to address these tissue specific differences. Recently,

Ding *et al.* (1999) described restrictions in the movement of *Brome mosaic virus* (BMV) in similar tissues, here referred to as bundle sheet tissue. The transport restriction was abrogated by a temperature effect and even though there is no evidence of an effect of temperature in the transport of the AMV mutants, the restrictions show similarities, indicating a similar cause of blockage.

However, AMV might also employ different movement mechanisms in different tissue types. Functional differences of the plasmodesmata between cortex cells and parenchyma cells might be the cause. It has been determined that the size exclusion limit (SEL) of plasmodesmata between sieve element-companion cell complexes is between 10 and 40 kDa (Kempers *et al.,* 1993; Kempers and Bel 1997; Imlau *et al.,* 1999) while the plasmodesmal SEL in parenchyma cells is ~ 1 kDa. Furthermore, a number of phloem proteins have been described to increase the plasmodesmal SEL to levels between 20-40 kDa in mesophyll cells upon microinjection (Balachandran *et al.,* 1997). Although the SEL of the cortex cells surrounding these vascular bundles and the possible effect of the phloem proteins on these cells is unknown, it is assumed that the cortex cells are involved in the marcomolecular trafficking to the vascular bundle (review Thompson and Schultz, 1999). This suggests that cortex cells have the ability to transport larger molecules and therefore might be able to transport the viral proteins from cell to cell.

Another appealing explanation is transport of RNA. Transport of a gene-silencing signal, presumably endogenous nucleic acids, was demonstrated to take place via the phloem (Palauqui *et al.,* 1997; Voinnet *et al.,* 1998). And in pumpkin the phloem protein CmPP16, a possible plant paralog to viral MP, was shown to be able to mediate transport of RNA (Xoconostle-Cázares *et al.,* 1999). It is tempting to use the characteristics of the vascular bundle to explain the observed translocation of the mutant virus through the cortex tissues. However, description of this tissue type, is unavailable at the moment. Tissues such as the cortex were troublesome in experimental analysis because of their inaccessibility and difficult microinjection procedures. New marker methods such as the green fluorescent protein (GFP) in combination with the mutants of AMV might provide good tools to study tissue dependent cell-cell communication and trafficking of macromolecules.

Concluding remarks

The cytological analysis of AMV CP and MP mutants in P12 protoplasts confirm the requirement of both an undisturbed tubule-forming capacity and whole virions for cell-to-cell movement in parenchyma cells. Still, local transport of the mutants deficient in cell-to-cell movement seems to occur in the cortex tissues surrounding the vascular tissue suggesting a different movement mechanism in these tissues.

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Chapter 4

The movement protein and coat protein of *Alfalfa mosaic virus* accumulate in structurally modified plasmodesmata.

Summary

In systemically infected tissues of *Nicotiana benthamiana*, *Alfalfa mosaic virus* (AMV) coat protein (CP) and movement protein (MP) are detected in plasmodesmata in a layer of 3 to 4 cells at the progressing front of infection. Besides the presence of these viral proteins, the plasmodesmata are structurally modified in that the desmotubule is absent and the diameter has increased drastically (almost 2-fold) when compared to plasmodesmata in uninfected cells or cells in which AMV infection had been fully established. Previously reported observations on virion-containing tubule formation at the surface of AMV-infected protoplasts suggest that AMV employs a tubule-guided mechanism for intercellular movement. Although CP and MP localisation to plasmodesmata is consistent with such a mechanism, no tubules were found in plasmodesmata of AMV-infected tissues. The significance of these observations is discussed.

Introduction

Systemic spread of plant viruses is achieved by movement of an infectious complex from cell to cell through plasmodesmata, followed by long distance transport through the vascular system. It has been established that plant viruses employ different mechanisms for movement of virions or viral RNA through plasmodesmata. Common characteristic of each mechanism employed is the inevitable modification of plasmodesmal gating or even drastic alteration of the plasmodesmal structures, which is established by one or more viral proteins, to enable such translocation (for recent reviews see Lucas and Gilbertson, 1994; Carrington *et al.*, 1996).

The mechanism of cell-cell movement of *Alfalfa mosaic virus* (AMV), a member of the *Bromoviridae*, is still not understood, although it has been established that the P3 movement protein (MP) (Van der Vossen *et al.*, 1995) and the coat protein (CP) (Van der Kuyl *et al.*, 1991; Van der Vossen *et al.*, 1994) of AMV are essential for virus movement. The MP of AMV has a RNA-binding capacity (Schoumacher *et al.*, 1992, 1994), and is transiently present in infected cells, localising to the middle lamella of plant cell walls of parenchymal and epidermal cells at the front of infection (Godefroy-Colburn *et al.*, 1986; Stussi-Garaud *et al.*, 1987). In AMV MP-expressing transgenic plants a slight increase in plasmodesmal macromolecular gating has been recorded (Poirson *et al.*, 1993). In the infection cycle of AMV the CP apparently has several functions. Besides encapsidation of the viral genome into quasi-isometric and bacilliform virions, the protein is also required for genome activation and RNA replication (Bol *et al.*, 1971; Jaspers, 1985; Quadt *et al.*, 1991). The way CP functions in cell-to-cell movement is not yet known. Recently, Kasteel *et al.* (1997) reported the formation of virion containing tubular structures at the surface of AMV-infected cowpea (*Vigna unguiculata*) and *Nicotiana benthamiana* protoplasts. These findings suggest a mechanism of tubule-guided movement of AMV virions through modified plasmodesmata, as has been described for several other plant viruses like comovirus (Van Lent *et al.*, 1990*a*), caulimovirus (Linstead *et al.*, 1988), nepovirus (Ritzenthaler *et al.*, 1995), tospovirus (Storms *et al.*, 1995) and geminivirus (Kim and Lee, 1992). To assess whether such a mechanism is indeed employed by AMV, further immunocytochemical and electron microscopical investigations were carried out in AMV-infected plant tissue.

Materials and Methods

Differential temperature inoculation (DTI)
From eight weeks old *Nicotiana benthamiana* plants the growing tip and all leaves were removed except the two fully-grown lower leaves and one small upper leaf (maximum length 3 cm). The lower leaves were inoculated with AMV strain 425 or water and then exposed to a differential temperature treatment where the inoculated leaves were kept in light at 25°C and the upper young leaf in dark at 6°C (method essentially as described by Dawson and Schlegel 1973). After 10 days, the young upper leaves were separated from the plant (this time point was defined as t=0 hrs)

and kept in a humid petridish under constant light at 25°C. The percentage of AMV-infected cells in the systemically infected leaves was established at 0, 12, 24 and 48 hrs post-DTI by isolation of protoplasts and immunofluorescent detection of AMV-CP and -MP (Van der Pelt-Heerschap *et al.,* 1987; Van Lent *et al.,* 1991).

Electron microscopy
Samples from systemically infected leaf tissues at 0, 12, 24 and 48 hrs post-DTI were fixed in a mixture of 2% (w/v) paraformaldehyde and 3% (w/v) glutaraldehyde in phosphate/citrate buffer (0.1 M $Na_2HPO_4.2H_2O$, 2.7 mM citric acid and 1.5 mM $CaCl_2$), pH 7.2 for 24 hrs at 4°C. The fixed tissues were then dehydrated and embedded in LR Gold as described by Van Lent *et al.* (1990*b*). Similar samples were subjected to freeze-substitution. For this, the tissues were fixed as described above and subsequently infiltrated with 1.0 M sucrose in water for 16 hrs at 4°C and cryo-fixed in liquid propane at -160°C in a Reichert-Jung KF80 cryofixation system. The frozen specimens were substituted with 0.5% glutaraldehyde in acetone at -90°C for 48 hrs in a Leica AFS freeze-substitution apparatus. The temperature was raised to -20°C at a rate of 4°C/hr. The acetone was substituted by ethanol for 1 hr, after which the specimens were infiltrated by LR Gold and the resin was polymerised under UV-light for 24 hrs at -20°C and 48 hrs at room temperature.
Alternatively, specimens were prepared for cryo-sectioning. Uninfected and systemically infected (24 hrs post-DTI) leaf tissue was aldehyde fixed and subsequently infiltrated with 2.0 M sucrose in water for 16 hrs at 4°C. The samples were fixed by plunging into liquid nitrogen (-196°C) and transferred to a cryo-ultramicrotome (Reichert FCS). Sections with a thickness of 110-150 nm were cut with a glass knife at -110°C and transferred in a drop of saturated sucrose solution to 150 mesh nickel grids with a formvar support film. Excess sucrose was washed out by incubation for 1 min on distilled water and the sections were negatively stained for 15 sec with 2 % (w/v) ammonium molybdate, pH 5.3, blotted and dried.

Immunogold labelling
Immunogold labelling on LR Gold or freeze-substituted resin was carried out as described by Van Lent *et al.* (1990*b*). Cryo-sections were first incubated on 0.1% (w/v) glycine in PBS for 20 min and then blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 45 min. Grids were then incubated on primary antibody for 16 hrs at 4°C, washed on drops of 0.1% (w/v) acetylated BSA (BSAc) (Aurion, Wageningen, the Netherlands) in PBS (6 x 5 min) and incubated with protein A-gold (7 nm or 10 nm gold particles; OD_{520}=0.05) for 1 hr. The grids were again washed with 0.1% (w/v) BSAc in PBS (4 x 5 min) and PBS (4 x 5 min) and incubated for 5 min on 1% glutaraldehyde in PBS. The sections were rinsed with water and stained with ammonium molybdate as described before.
Unless stated otherwise, all incubations were done at room temperature.

Microscopy and measurements
Sections were viewed with a Philips CM12 transmission electron microscope. Measurements of the diameter of plasmodesmata were done on-line using AnalySIS Docu software (Soft-Imaging Software, GmbH). Only plasmodesmata in palisade and

spongy parenchyma cells of the leaf tissue were measured. The plasmodesmal diameter was defined as the average of two measurements taken at the widest and narrowest part of the plasmodesmal structure.

Results

As cell-to-cell movement of AMV is a transient process, it was essential to obtain systemically infected tissues with cells in an early and synchronised stage of infection for structural and immunocytochemical analyses of virus movement phenomena. Such tissues were obtained by differential temperature inoculation (DTI) of *N. benthamiana* plants with AMV. During the differential temperature treatment of plants, virus replicates in the mechanically inoculated lower leaves, which are kept at high temperature and in light. Progeny virus is transported (through the vascular system) to the young not fully developed top leaves that are kept at low temperature and in the dark, a condition that is nonpermissive for virus replication. After several days, when enough inoculum virus has moved from the lower leaves into the young top leaves, these leaves are separated from the plants and transferred to a condition which is permissive for virus replication (t=0 of systemic infection) and virus synthesis begins simultaneously in all invaded cells (Dawson and Schlegel, 1975).

After DTI the young top leaves were separated from the plants and the percentage of infected mesophyll cells was established at 0, 12, 24 and 48 hrs by isolation of protoplasts and immunofluorescent detection of the CP immediately after isolation and after further incubation of the protoplasts for 16 hrs. The results are summarised in Table 1 and show that there is a sixteen-fold increase of infected mesophyll cells between 12 and 24 hrs post-DTI. Prolonged incubation of protoplasts for 16 hrs and subsequent detection of CP also revealed the number of cells which were invaded by AMV at the moment of sampling, but in which infection was not yet sufficiently established to allow immunofluorescent detection of CP. These results show that virus spread is most extensive between 12 and 24 hrs post-DTI.

Table 1. *Percentage AMV-infected* N. benthamiana *protoplasts isolated from DTI leaves, incubated in light conditions for 0, 12, 24 and 48 hrs. The infection percentage was determined by immunofluorescent detection of CP directly upon isolation of the protoplasts and after 16 hrs incubation of the protoplasts in culture medium.*

hrs post-DTI	% infected protoplasts (CP detection)	
	upon isolation	after 16 hrs incubation
0	0	<1
12	2	4
24	32	45
48	61	77

Occasionally, in protoplasts isolated from leaves at 24 hrs post-DTI and incubated further for 16 hrs, fluorescent tubular structures were observed at the cell surface after treatment with antiserum against the AMV-MP (data not shown). The percentage of protoplasts showing this phenomenon varied strongly with each experiment, ranging from 0.1 to 10%.
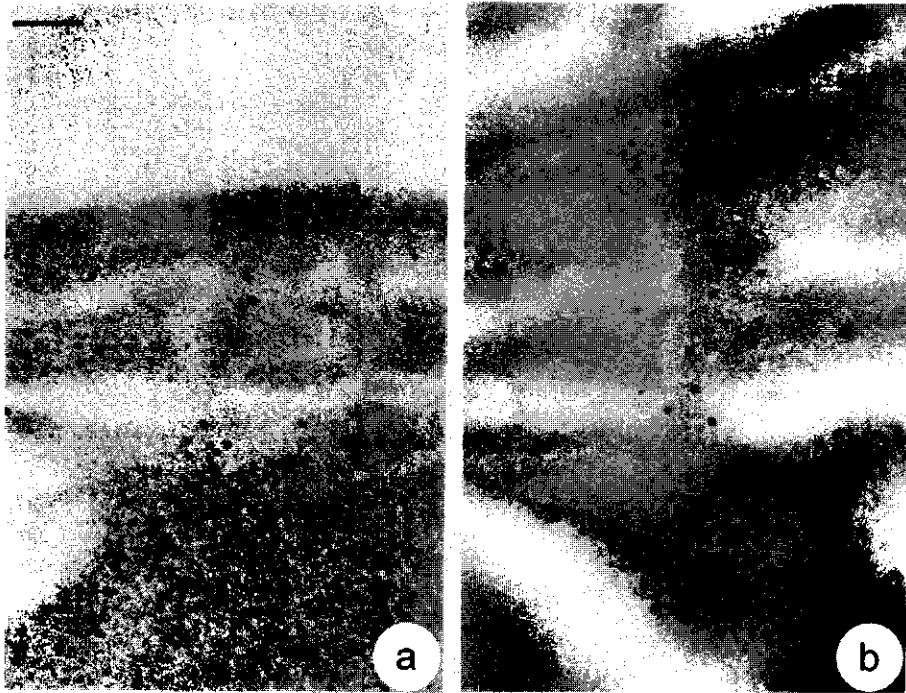
Immunogold detection of AMV CP and MP

For electron microscopical analysis samples from leaves at 24 hrs post-DTI were used as it was expected that these tissues contained sufficient mesophyll cells in early stages of infection to allow convenient detection of movement phenomena. Infected foci, and in particular the border area between infected and uninfected tissue, were identified in cross sections of AMV-infected leaves by immunogold detection of CP. In these cells CP was predominantly localised in the cytoplasm. Figure 1 schematically represents a cross section of a DTI-infected leaf containing the border area between AMV-infected cells and uninfected cells. In most AMV-infected cells (zone b and c) CP was localised in the cytoplasm. However, a layer of approximately 3 to 4 infected cells could be identified, adjacent to uninfected cells (zone b), in which the CP was also present in plasmodesmata (Figure 2a). CP was never detected in
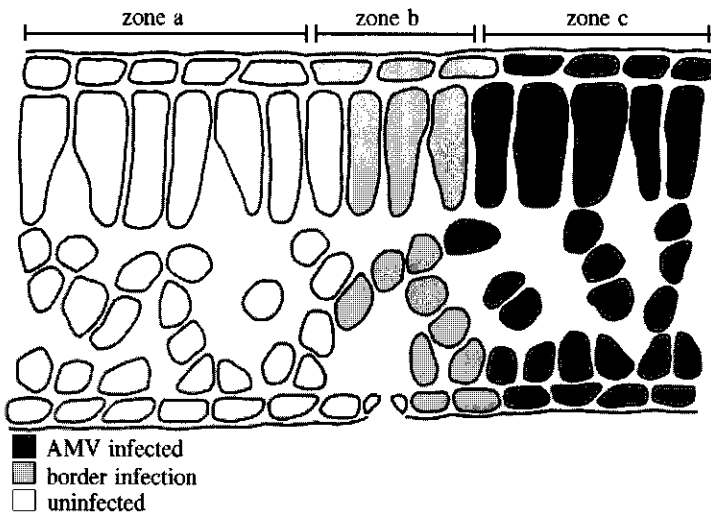
Figure 1. *Schematic representation of a cross section of AMV-infected leaf tissue. Based on the immunogold labelling of AMV CP and MP, three zones can be distinguished. Zone (a) containing uninfected cells shows no significant labelling, zone (b) containing recently infected cells shows gold labelling of CP and MP in plasmodesmata and limited CP labelling in the cytoplasm and zone (c) shows no labelling of plasmodesmata but high amounts of CP in the cytoplasm.*

plasmodesmata between uninfected cells (zone a). In sections of the same tissue the MP was exclusively localised in plasmodesmata of AMV-infected cells within this front of infection (zone b; Figure 2 b), and not in plasmodesmata of other AMV-infected cells in zone c or in uninfected cells.


Figure 2. *Immunogold detection of AMV CP (a) and MP (b) in plasmodesmata of N. benthamiana mesophyll cells at the border of AMV infection (zone b, Figure 1). Bar represents 50 nm.*

Analysis of structural modifications of plasmodesmata within the infection front
In freeze-substituted tissue, it was noted that the diameter of CP- or MP-labelled plasmodesmata in cells at the front of infection (zone b) was larger than the diameter of unlabelled plasmodesmata in fully infected (zone c) or uninfected cells (zone a). Figure 3 shows examples of plasmodesmata illustrative for these three zones. In the uninfected tissue (zone a) the plasmodesmata are narrow (Figure 3 a,b) like the plasmodesmata from fully-infected cells located behind the infection front (zone c, Figure 3 g,h). Between cells within the infection front (zone b, Figure 3 c,d,e,f) enlarged plasmodesmata were observed.

To certify an increase in plasmodesmal diameter during movement of AMV, the diameter was determined in mesophyll cells of healthy tissue (zone a, Figure 4a) and randomly in the 3 zones of the AMV-infected tissue at 24 hrs post-DTI (Figure 4b). Furthermore, the diameter of plasmodesmata in cells within the front of infection (zone b) and gold labelled with anti-CP or anti-MP, was measured (Figure 4c) as well
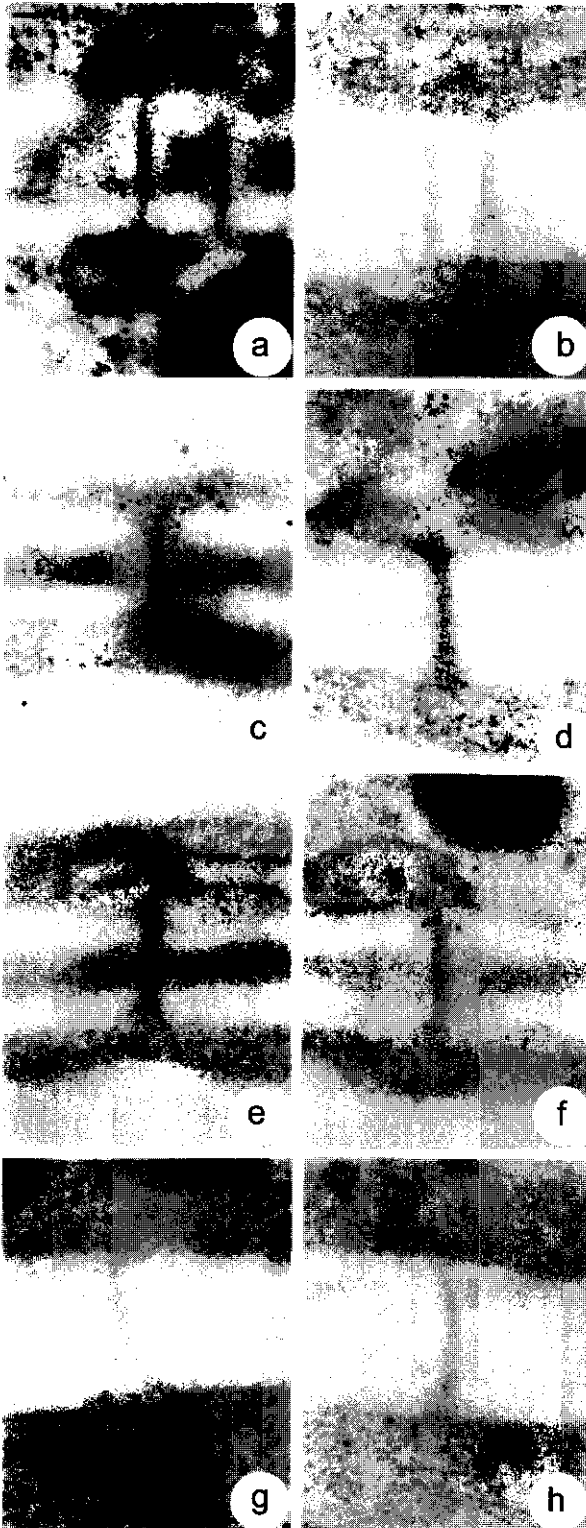
Figure 3. *Plasmodesmata of N. benthamiana mesophyll cells. Sections were gold labelled with anti-CP (a to d, g and h) and anti-MP (e and f). (a,b) Plasmodesmata located between uninfected tissue (zone a, Figure 1). (c,d,e,f) Plasmodesmata located between cells within the border of infection (zone b, Figure 1). (g,h) Plasmodesmata located between cells in which AMV infection had been established for a longer period (zone c, Figure 1). Note that the diameter of the dark stained core of the plasmodesmata in c to f is enlarged compared to that of the plasmodesmata in a,b and g,h. Bar represents 50 nm*
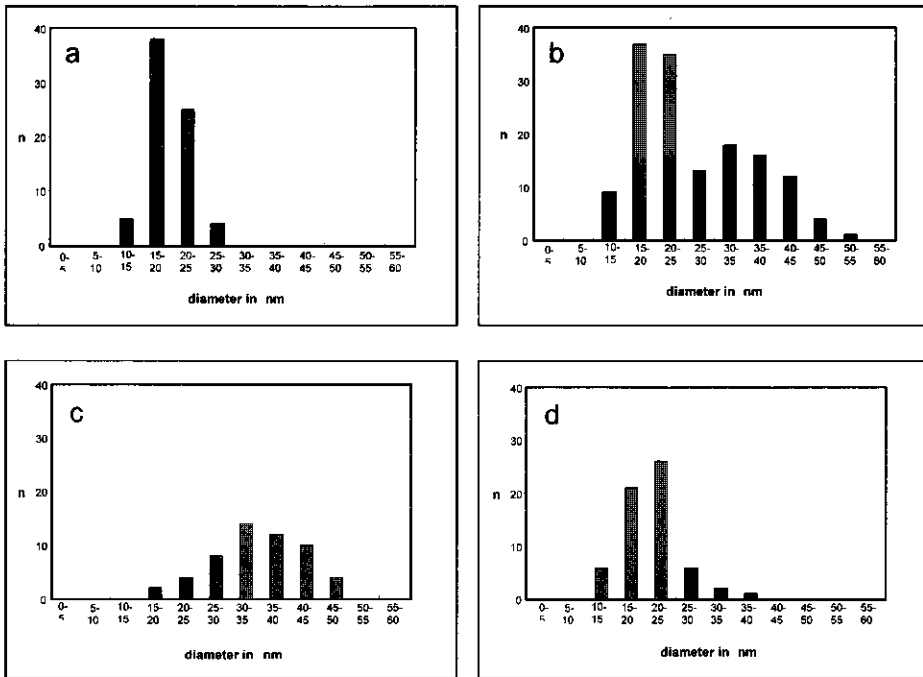
Figure 4. *Distributions of the plasmodesmal diameters in* N. benthamiana *mesophyll cells. Measurements were taken from plasmodesmata: a) in uninfected cells (zone a, Figure 1)(χ=19.5 ± 3.1 n=72), b) randomly (zones a,b and c, Figure 1)(χ=26.5 ± 9.6 n=144), c) from plasmodesmata gold labelled with CP or MP (zone b, Figure 1)(χ=36.1 ± 9.0 n=56) and d) in fully infected tissue (zone c, Figure 1) (χ=21.2 ± 4.8 n=62). The number of plasmodesmata measured is n.*

as the diameter of plasmodesmata in fully AMV-infected cells (zone c, Figure 4d). The measurements were done on thin sections of freeze-substituted leaf tissue and the diameter was defined as the average of two measurements taken at the widest and smallest part of the plasmodesmal structure. As no fine structural detail could be discerned within the plasmodesmata, the width of the plasmodesma was established by measuring the width of the dark stained core (as shown in Figure 3).

Measurements in healthy control tissue showed a normal distribution in plasmodesmal diameter between 10 to 30 nm with an average of 19.5 ± 3 nm (n=72) (Figure 4a). Similar measurements done randomly in the three zones of AMV-infection, showed a very different distribution in plasmodesmal diameters (Figure 4b). One population of plasmodesmata showed a diameter distribution similar to that of healthy tissue. However, others showed a drastic increase in their diameter. Measurement of plasmodesmata specifically within the front of infection, and identified by CP or MP gold labelling showed a normal distribution in diameters between 15 and 50 nm with an average diameter of 36.1 ± 9 (n=56) (Figure 4c). The diameter of unlabelled

plasmodesmata present in AMV-infected cells behind this front of infection (zone c) had decreased again to an average of 21.2 ± 4.8 (n=62) (Figure 4d).

It has to be taken into account that part of the variation in plasmodesmal diameter may be due to a variety in portions of the three dimensional plasmodesmal structure that are included in a thin section.

As no fine structural details of plasmodesmata could be discerned in thin sections of freeze-substituted AMV-infected leaf tissue, similar samples were cryo-fixed and cryo-sectioned to achieve better structural preservation. Gold labelling of CP or MP on plasmodesmata of these cryo-sections was, for unknown reasons, not successful. However, random measurements of the plasmodesmal diameter in uninfected and AMV-infected samples revealed similar distributions as described above (data not shown). It was noted that the diameter of plasmodesmata measured in cryo-sections in general was much larger than that measured in sections of freeze-substituted tissue (for plasmodesmata in healthy tissue resp. 33.9 ± 3.8 and 19.5 ± 3.1 nm). This could only be attributed to the very different preparative techniques used. In cryo-sections the structural detail of plasmodesmata was much improved when compared to freeze-substituted samples. Figure 5a shows two plasmodesmata from uninfected tissue with a normal size and substructure, clearly containing the plasma membrane and desmotubule. In plasmodesmata with an enlarged diameter, only present in tissue in which the AMV infection is proceeding, the desmotubule was consistently absent. Also, in a limited number of cases, the ultrastructure of the modified plasmodesmata was preserved good enough to visualise virions lined up within a tubule-like structure in the plasmodesmal pore (Figure 5 b,c).
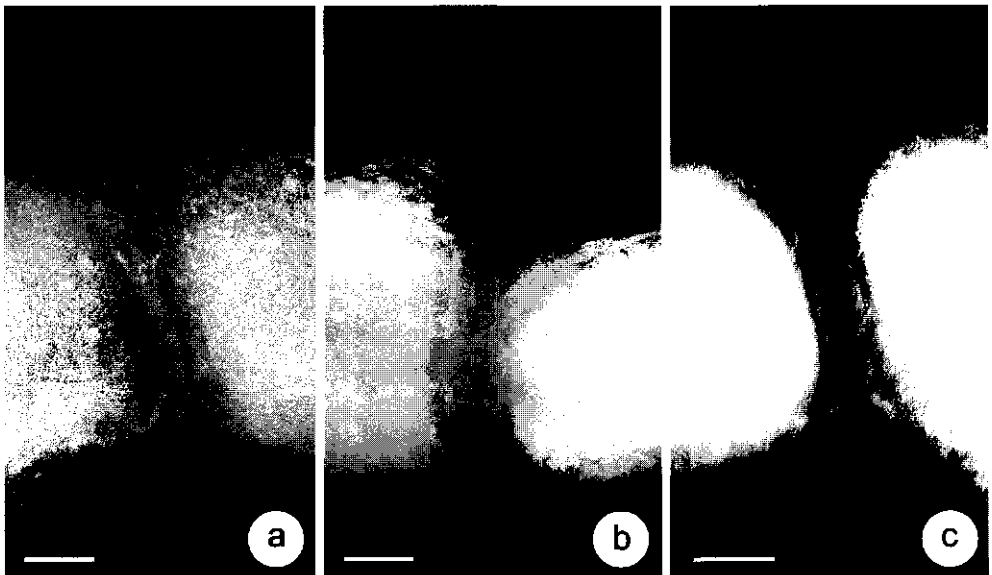
Figure 5. *Electron micrographs of plasmodesmata in cryo-sections of N. benthamiana mesophyll cells a) uninfected and b,c) in AMV-infected cells at the front of infection (zone b, Figure 1). Bar represents 50 nm.*

Discussion

Our results show that only in a layer of 3 to 4 cells at the front of AMV-infection in *N. benthamiana* leaves, plasmodesmata are structurally modified and contain CP and MP. Behind this infection front in cells where AMV-infection has been fully established at earlier stages, no modified plasmodesmata were observed anymore. The modification of plasmodesmata comprises the removal of the desmotubule and an increase in diameter, from average 20 nm in uninfected or fully infected cells, to 36 nm in cells at the front of infection.

Godefroy-Colburn *et al.* (1986, 1990) and Stussie-Garaud *et al.* (1987) reported the transient presence of MP in epidermal and parenchymal cells at the front of AMV-infection. The authors localised the MP predominantly to the middle lamella of the cell walls and only occasionally observed some association of MP and CP with plasmodesmata (Godefroy-Colburn *et al.*, 1990). In our experiments we have never found MP in the middle lamella of cell walls, but could only localise MP in plasmodesmata at the infection front. Also the CP was frequently found in plasmodesmata of the same cells. The discrepancy between these observations cannot easily be explained.

Recently, the formation of long tubules at the surface of AMV-infected protoplasts, consisting of MP and occluding AMV virions was reported (Kasteel *et al.*, 1997). These results suggest that AMV employs a tubule-guided movement mechanism for intercellular translocation through plasmodesmata, comparable to that of the comoviruses (Van Lent *et al.*, 1990*a*, 1991). The localisation of CP and MP to plasmodesmata is consistent with such a mechanism. Furthermore, in a few protoplasts isolated from DTI-infected tissues tubule formation was observed after prolonged incubation of these protoplasts in culture medium. These cells were probably in an early stage of infection and as such in a state comparable with *in vitro* inoculated protoplasts (Kasteel *et al.*, 1997). A structure resembling a tubule within a plasmodesma and containing CP was reported by Godefroy-Colburn *et al.* (1990). However, no virions were discerned inside this tubule. During scrutinous inspection of the cryosections, virion-containing tubules in plasmodesmata of AMV-infected tissues were observed although at a low rate. As the presence of MP in plasmodesmata is transient, occurring only in few cells at the front of infection, tubule formation may be difficult to discern. Considering the transient nature of AMV MP in cells, it is not likely that long tubules are formed as are observed in *Cowpea mosaic virus* (CPMV)-infected cells (Van Lent *et al.*, 1990*a*, 1991). In the latter equimolar amounts of the CPMV MP and CP are synthesised throughout the infection cycle, enabling constant further assembly of tubules in plasmodesmata, resulting in long tubules eventually packed in cell wall material and sticking out into the neighbouring cell (Van der Scheer and Groenewegen, 1971). In AMV-infected cells at the infection front, tubules may be restricted to the interior of the plasmodesma.

Modification of plasmodesmata is essential to allow assembly of a movement tubule. Like with CPMV, the desmotubule appears to be removed from plasmodesmata in AMV-infected cells that are implicated in virus movement. An increase in plasmodesmal diameter has never been noted before for CPMV or any other virus employing a similar movement mechanism, although this aspect may have been

overlooked in other studies. Adaptation of the plasmodesmal diameter may also be a prerequisite to allow assembly of an AMV movement tubule and as our study shows this adaptation is transient. How this sizing of plasmodesmata is achieved is unclear, but similar mechanisms may be involved that regulate the opening and closure of plasmodesmata in uninfected plant cells (Schulz 1995).

Acknowledgement

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Chapter 5

Novel plasmodesmata induced in parenchyma cells at the border of *Alfalfa mosaic virus* infection.

Summary

For intercellular movement of *Alfalfa mosaic virus* (AMV), plasmodesmata are structurally modified as shown previously (Van der Wel *et al.*, 1998). The modification comprises loss of the desmotubule, a twofold increase in diameter and the presence of the viral movement and coat proteins. Plasmodesmata modified by the virus can only be detected in cells at the infection border as they disappear in fully infected cells. During thorough examination of the AMV infection border, it was noted that the number of plasmodesmata present in cells at the expanding infection front, appeared to be higher than in uninfected tissue. Therefore, the number of plasmodesmata per µm of cell wall was determined in different infection zones of systemically infected *N. benthamiana* leaf tissue. In cells at the border of infection, the average number of plasmodesmata per µm of cell wall increased by a factor three to four compared to the plasmodesmal frequency in uninfected or fully infected cells. Apparently, during initial stages of AMV infection novel intercellular channels are formed, which appear to be genuine plasmodesmata as they share biochemical properties such as the presence of three plasmodesma associated components. These observations suggest that either virus infection induces the formation of novel channels for viral cell-to-cell movement or the plant cells respond on the blockage of the pre-existing plasmodesmata by forming new plasmodesmata to restore and maintain intercellular communication.

Introduction

Plasmodesmata are intercellular channels that maintain symplastic domains and provide a pathway for cell-to-cell trafficking of (macro)molecules that are required for plant growth and development (McLean *et al.*, 1997). At the same time plasmodesmata are utilised by plant viruses for their cell to cell spread to establish systemic infection (Carrington *et al.*, 1996). In general the physical and chemical properties of plasmodesmata exclude the flow of molecules larger than approx. 1 kilodalton (kDa) (Goodwin, 1983). To achieve movement of the large viral genome or even the virus particle, plasmodesmata must be modified, a process involving in all cases a virus-encoded movement protein (MP). The type of modification of plasmodesmata depends on the type of virus infecting the plant tissue and may range from modification of the size exclusion limit without noticeable structural adaptations (e.g. *Tobacco mosaic virus* (TMV), Wolf *et al.*, 1989, Tomenius *et al.*, 1987) to severe structural modification which includes the removal of the desmotubule and insertion of a movement tubule made up of the viral MP for transport of mature virions (e.g. *Cowpea mosaic virus* (CPMV), Van Lent *et al.*, 1991).

There is increasing evidence that *Alfalfa mosaic virus* (AMV) employs the latter, tubule-guided, movement mechanism. It has been shown that the viral MP and the coat protein (CP) are essential for systemic movement (Van der Kuyl *et al.*, 1991; Van der Vossen *et al.*, 1994, 1995) and that AMV infected protoplasts form virion-containing tubules at their surface (Kasteel *et al.*, 1997). Plasmodesmata in AMV-infected plant mesophyll cells contain the viral MP and CP and are structurally modified. The modification of plasmodesmata comprised loss of the desmotubule and an increase in diameter from average 20 nm to average 36 nm (Van der Wel *et al.*, 1998). The insertion of a tubular structure was suggested by Godefroy-Colburn *et al.* (1990) and Van der Wel *et al.* (1998) although the visualisation of the tubule or the virions herein appeared to be rather difficult. Also the rapid turnover makes it difficult to discern the tubules since the modification the plasmodesmata is indeed a transient process, occurring only within a layer of 3-4 cells at the advancing front of infection (Van der Wel *et al.*, 1998).

Apparently, after their involvement in the process of virus movement in cells at the front of infection, the modified plasmodesmata disappear or restore their original properties, as modified plasmodesmata are no longer found in fully infected cells behind the infection front. Also for TMV it was nicely shown by microinjection studies that the increase in the plasmodesmal size exclusion limit is a transient process, specifically occurring in the expanding infection sites (Oparka *et al.*, 1997). The transient character of plasmodesmal modification would imply a dynamic character of plasmodesmata; i.e. the plant cell would have the capacity to either restore modified plasmodesmata or to eliminate them.

During thorough electron microscopic examination of cells within the AMV infection border, it became apparent that the number of plasmodesmata in these cells had increased when compared to uninfected cells and fully infected cells. These observations and the transient character of the plasmodesmal modification give rise to the idea that new plasmodesmata are formed as a response to virus infection. It is evident that plant cells have the capacity to form new plasmodesmata in non-dividing

walls, as has been described in several studies on graft interfaces (Jeffree & Yeoman 1983, Binding *et al.,* 1987, Kollmann and Glockmann 1985 and 1991), interfaces between host and parasite cells (Tainter 1971, Dell *et al.,* 1982), in elongating cells (Schnepf and Sych 1983, Seagull 1983, Zhu *et al.,* 1998) and between carpels (Van der Schoot *et al.,* 1995).

In this chapter, we provide evidence for the induction of new plasmodesmata in cells at the front of AMV infection. The new plasmodesmata might be those actively involved in movement of AMV or alternatively, those to maintain cell-cell communication of the plant.

Methods

Preparation of plant tissue for electron microscopy

Eight weeks old *N. benthamiana* plants infected with AMV were used 7 days after inoculation. At this time, the upper not fully expanded leaves showed two zones, a symptomatic basal part and a symptomless top part. Three different samples were taken from these leaves, one set of samples from the symptomatic (and fully infected) basal part of the leaf, one set of samples from the uninfected top part of the leaf and on set of samples from the intermediate zone. The samples were prepared for electron microscopical analysis by using freeze substitution as previously described by Van der Wel *et al.* (1998). The samples used for immuno-labeling using plasmodesmal probes, were pre-fixed in 4% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde in phosphate citrate buffer, pH 5.3 (0.1 M $Na_2HPO_4 \cdot 2H_2O$, 2.7 mM citric acid) and after cryo-fixation water was substituted at $-90°C$ by 1% (w/v) osmiumtetroxide (OsO_4) in aceton. Sections with a thickness of 100 nm were cut with a Reichert Ultracut S.

Determining the number of plasmodesmata

To establish the number of plasmodesmata, cross-sections of the leaf tissue were made and plasmodesmata were counted in stretches of cell walls only between palisade parenchyma cells to avoid fluctuations in number of plasmodesmata due to differences in cell types. At the same time the length of the cell wall stretch was measured and the number of plasmodesmata per length of cell wall was calculated.

The measurements were done in several different sections of five different borders of infection which were identified by immunogold labeling using an antiserum against AMV-CP (Van der Wel *et al.,* 1998; Van Lent *et al.,* 1990). Cells in the vicinity of the border of infection were not used for the determination of the plasmodesmal frequency of the uninfected or fully infected zones. Within the border of infection, only walls of cells that contained at least one CP-labelled plasmodesma, were used for the determination of the plasmodesmal frequency. Only those plasmodesmata in a section were counted that were visible for at least half their length. Plasmodesmata appearing in cross-section were not counted. The statistical analysis was done using the Kruskal-Wallis one way analysis of variance on ranks ($P < 0.05$).

Immunogold labeling
Immunogold labeling was performed as described by Van Lent *et al.* (1990).
Antisera were diluted in 1% (w/v) BSA in PBS. Antiserum against the AMV MP and
CP were a gift from Prof. J. Bol (Van Pelt-Heerschap *et al.*, 1987) and diluted 1:1000
times. Antiserum against callose was obtained from Genosys Biotechnologies Inc. UK
and used in a 1:1000 dilution. The monoclonal antibodies JIM 64 and JIM 67 were a
gift from Prof. K. Roberts and used in a dilution of 1:50. Prior to the labeling with
anti-callose, the sections were blocked with 5% BSA in PBS for 1h. All other sections
were blocked in 1% BSA in PBS for 30 min.
For the double labeling sections were blocked, incubated for 1 h with antiserum
against AMV-MP and subsequently incubated for 1h with goat anti-rabbit IgG
conjugated to 6 nm gold. The sections were again blocked in 1% BSA in PBS for 30
min and incubated with the second antiserum against JIM64 or JIM67 for 1 h and
subsequently, after washing, with goat anti rat IgG conjugated to 10 nm gold.

Results

Number of plasmodesmata in AMV-infected cells
As shown previously (Van der Wel *et al.*, 1998), three zones could be identified in
mesophyll tissue of *N. benthamiana* leaves systemically infected with AMV. One
zone comprising still uninfected cells, one zone of approximately 3-4 cells in which
AMV-infection just had been established and characterised by the presence of viral
MP and CP in modified plasmodesmata, and a zone of cells in which AMV-infection
had been fully established and in which modified plasmodesmata were absent. In five
different leaf samples including the border of AMV-infection, plasmodesmata were
counted in stretches of cell wall between palisade parenchyma cells in each of the
three zones as is illustrated in Figure 1. The results were expressed as the average
number per 20 μm of cell wall and are summarised in Table 1.

Table 1. *The number of plasmodesmata (PD) present in three zones of the AMV*
infection per 20 μm cell wall. The calculated standard error (SE), the total length of
cell wall examined and the total number of plasmodesmata counted are also given.

Zone	Number PD	Total cell wall in μm	PD per 20 μm	SE
Uninfected	78	912	1.7	0.8
Border infection	60	251	4.8	0.7
Infected	24	404	1.2	0.5

a

c
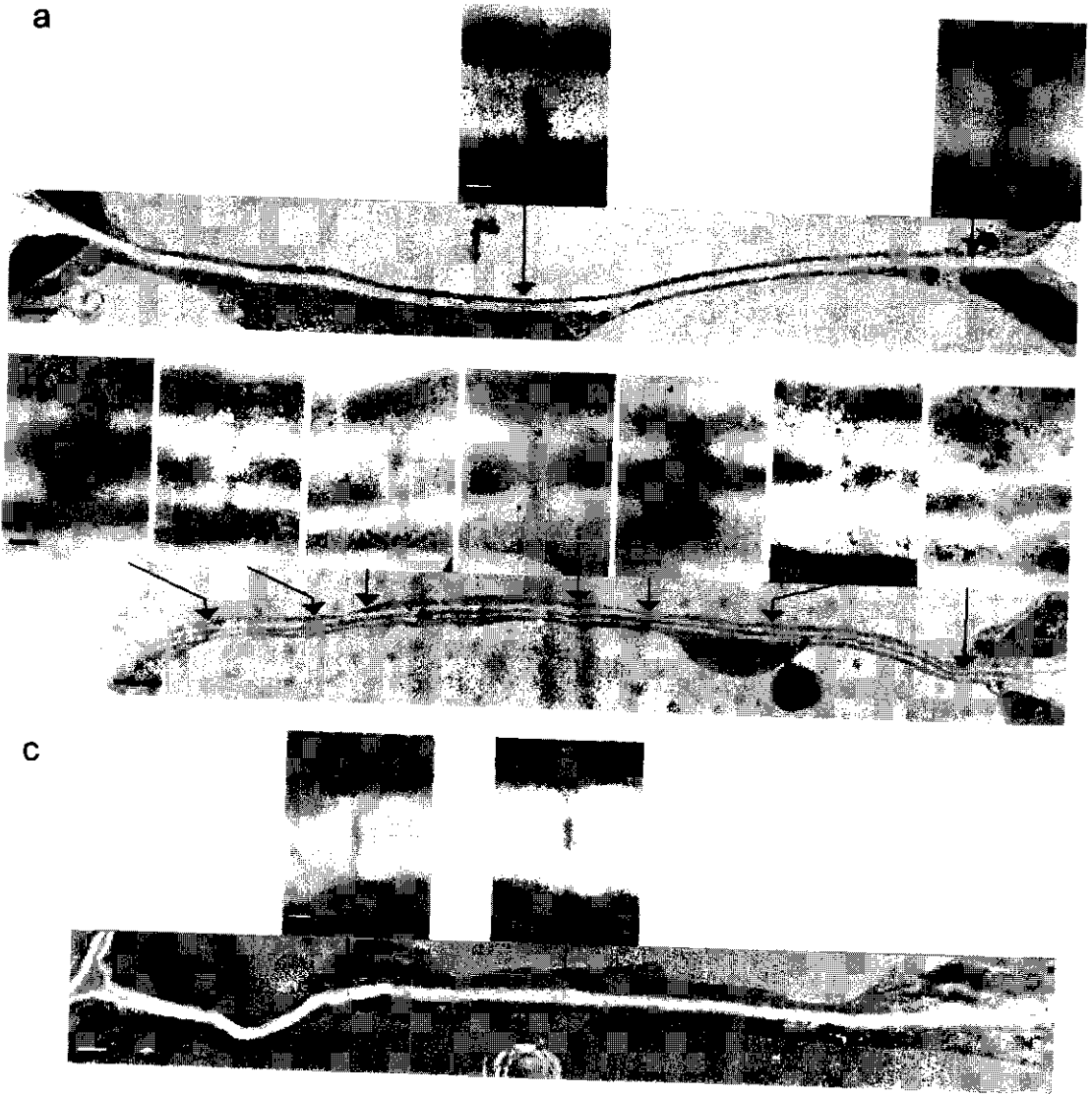
Figure 1.
Electron micrographs of vertical cell wall interfaces of palisade parenchyma cells and enlargements of the herein present plasmodesmata from (a) uninfected leaf tissue, (b) cells at the front of AMV-infection and (c) fully infected tissue. Cytoplasmic immunogold labeling of AMV-CP indicate AMV-infected cells. Bar represents 1 µm in the overviews and 100 nm in the enlarged micrographs.

Within the uninfected zone and fully infected zones a total of 1316 μm of cell wall was examined showing an average of 1.7 and 1.2 plasmodesmata per 20 μm respectively. However, within the border of infection an average of 4.8 plasmodesmata per 20 μm of cell wall was determined from countings in a total of 251 μm cell wall. This means that in cells that are actively involved in AMV-movement, the number of plasmodesmata is three- to four-fold higher than in uninfected and fully infected cells. Statistical analysis of these data shows no significant difference in number of plasmodesmata in uninfected and fully infected tissue, but a significant increase in cells within the infection border. This demonstrates that novel plasmodesmata are induced in cells actively involved in AMV movement.

The question now raises if all plasmodesmata at the infection front are involved in the movement of the virus and therefore ultra-structurally modified or if plasmodesmata available for plant cell-cell communication are still present. Based on unpublished data from the study of Van der Wel *et al.* (1998), the percentage plasmodesmata on the infection border which were immunogold labelled with antisera against the viral proteins, was determined to be 30%. This percentage is probably not the actual amount of modified plasmodesmata since immunogold labeling of plasmodesmata hardly ever covers all plasmodesmata due to the fact that only the antigens on the section surface are reachable for the antiserum. Likewise, labeling of plasmodesmata using antisera against actin, ubiquitin and a putative plasmodesmata-associated protein, resulted in only 8-28 % labelled plasmodesmata (Blackmann *et al.,* 1998; Ehlers *et al.,* 1996; White *et al.,* 1994). Considering these variable results, immunogold labeling of plasmodesmata appears to be an unreliable method for the discrimination of modified plasmodesmata from unmodified plasmodesmata.

The alternative method for the determination of the percentage modified plasmodesmata is to use the diameter of plasmodesmata. From measurements at the border of infection, it was determined that on average 57% of the plasmodesmata had an increased diameter. For these counts, only plasmodesmata were used which were entirely present in the ultra-thin section, excluding the ones which are grazed. However, a number of modified or viral protein containing plasmodesmata will not have been included in these counts because there is an overlap in the average diameter of the unmodified and viral protein containing plasmodesmata (van der Wel *et al.,* 1998). Therefore, the exact percentage of modified plasmodesmata will be slightly higher. Still it is evident that part of the plasmodesmata on the infection border is unmodified demonstrating that plasmodesmata available for the host's cell-cell communication remain present.

Identical immuno-labeling on modified and unmodified plasmodesmata
As the previous results show a more than three-fold increase in number of plasmodesmata in plant cells at the infection front, the question arises whether the newly formed channels not only resemble plasmodesmata in structural, but also in biochemical properties. For this, immunogold labeling using antibodies known to associate with plasmodesmata was performed. The monoclonal antibodies JIM64 and JIM67 are raised against proteins isolated from cell wall extracts from maize and specifically recognise one or more antigens associated with the plasmamembrane part
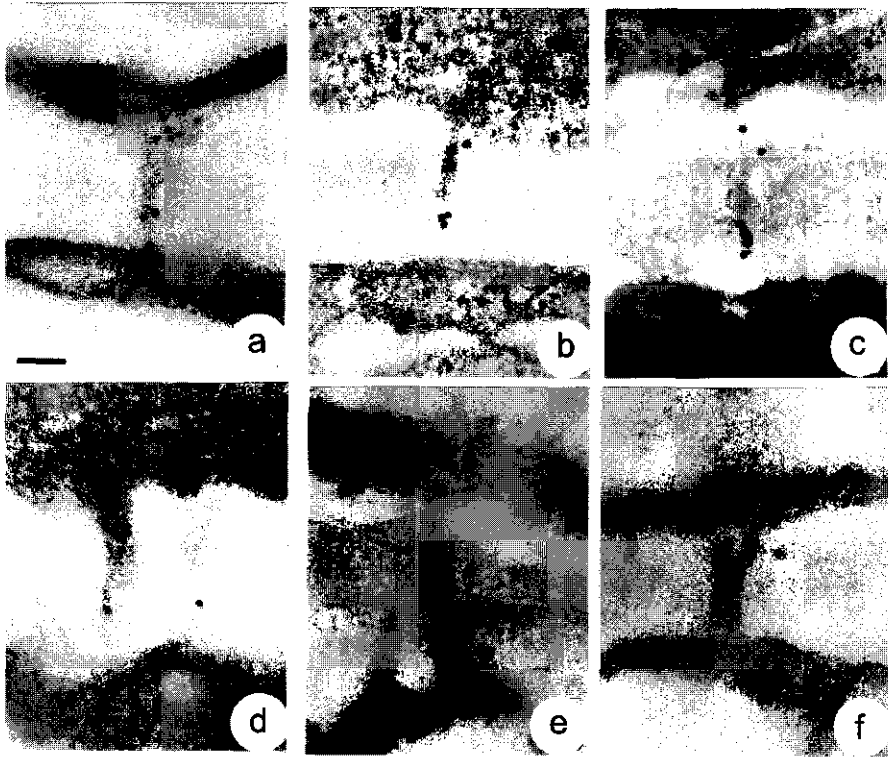
Figure 2.
Immunogold labeling of callose on plasmodesmata in uninfected cells (a,b,c) and on plasmodesmata modified by the virus in cells at the border of AMV infection (d,e,f). Bar represents 100 nm.

of plasmodesmata in *N. clevelandii* (Waigmann *et al.*, 1997). The polyclonal antibody against callose is known to immuno-localise to the neck region of plasmodesmata (Rinne and Van der Schoot, 1998).

In uninfected *N. benthamiana* tissue, callose was specifically detected in this region (Figure 2 a-c). Also in cells within the border of AMV infection, plasmodesmata were labelled in a similar fashion with no observable difference in labeling between unmodified and modified plasmodesmata (Figure 2 d-f).

In uninfected cells, immunogold labeling was observed on 50% of the plasmodesmata when using the monoclonal antibodies JIM64 or JIM67 (Figure 3a and d resp.). In cells within the border of infection similar results were obtained. Double gold labeling with JIM64 or JIM67 tagged by 10 nm gold particles and the AMV-MP tagged by 6 nm gold particles showed plasmodesmata containing both components (Figure 3 b, c and e), indicating that plasmodesmata which were actively involved in AMV-movement still contained the plasmodesma associated components. In controls consisting of sections labelled in a similar fashion but in which the second primary antibody (JIM64 or JIM67) was omitted, no double labeling of plasmodesmata was observed (Figure 3 f).
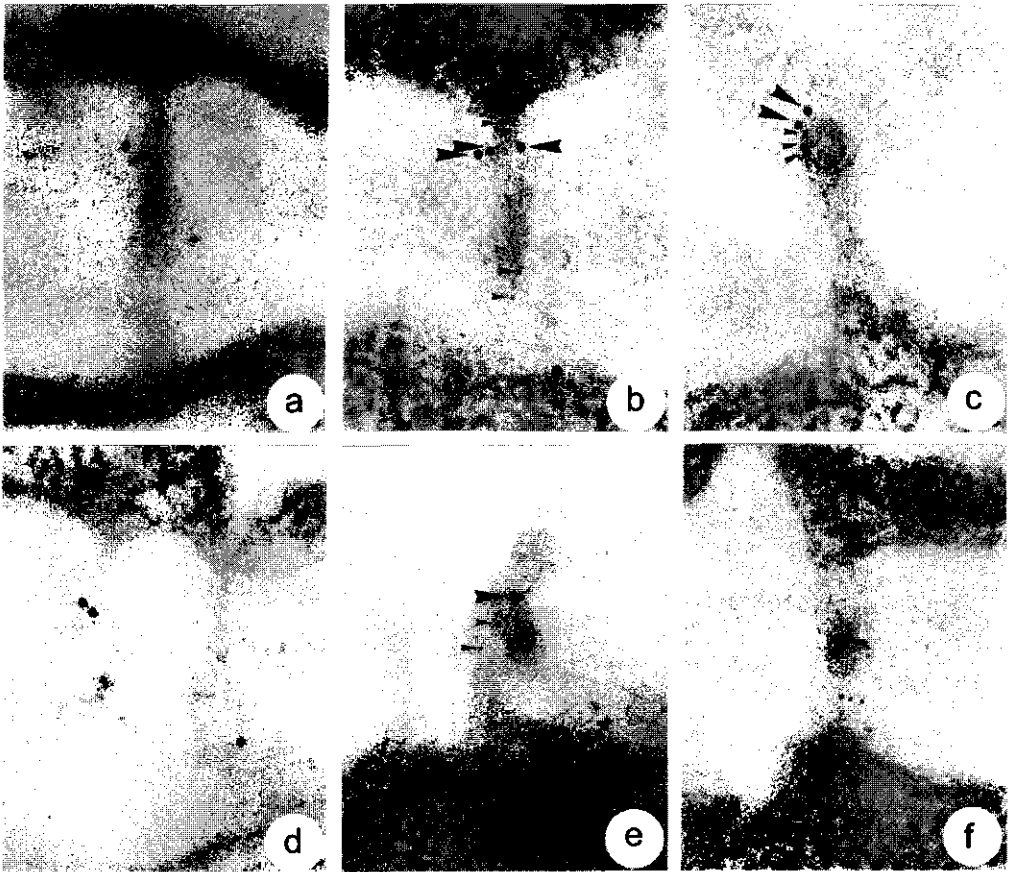
Figure 3.
Immunogold localisation of plasmodesmal components recognised by monoclonal antibodies JIM64 (a) and JIM67 (d). Double labelling using antisera JIM64 (b,c) JIM67 (e) marked by 10 nm gold particles and antiserum against AMV MP marked by 6 nm gold particles shows the presence of both components in plasmodesmata at the front of infection. Control labelling using only antiserum against the AMV MP and both GARat 10 nm and GARabbit 6nm gold (f) shows no double labelling. Bar represents 100 nm.

Discussion

Our results show a significant, at least three-fold increase of the number of plasmodesmata in *N. benthamiana* mesophyll cells within the advancing front of AMV infection. Apparently, as a result of this infection new channels across the cell walls are formed in these cells, which resemble plasmodesmata in structural properties and which indeed react with different plasmodesma specific probes. It was already

previously shown (Van der Wel *et al.,* 1998) that the structure of plasmodesmata in mesophyll cells within the front of infection is modified to allow transport of AMV virions. This modification comprises the removal of the desmotubule and enlargement of the plasmodesmal diameter and is transient as modified plasmodesmata are only found within a layer of three to four cells at the front of infection. As the number of plasmodesmata in AMV-infected cells outside this front of infection is not significantly different from the number recorded in uninfected cells, the increase in number of plasmodesmata appears to be equally transient as is the structural modification and coincides with active AMV-movement. These results may be interpreted in two ways. Upon AMV infection either existing plasmodesmata are modified to facilitate movement of virions and the plant cell induces new plasmodesmata to restore its intercellular communication or, alternatively, the virus evokes a mechanism by which new channels are formed to specifically facilitate its intercellular movement.

Little information is available on the dynamics of plasmodesmata in plant mesophyll tissues. Our studies were done on samples from young expanding *N. benthamiana* leaves and it has been established for young cells from *Zea mays* and *Arabidopsis thaliana* roots (Seagull 1983; Zhu *et al.,* 1998) and *Sphagnum palustre* leaflets (Schnepf and Sych, 1983) that new plasmodesmata can be formed before or during elongation. Also during the transition of leaves from sink to source a rapid increase in the number of plasmodesmata in minor veins from *Cucumis melo* and *Curcurbita pepo* has been reported (Volk *et al.,*1996). It is therefore not unlikely that the infected plant cell, upon modification of its existing plasmodesmata, responds by forming new plasmodesmata to restore intercellular communication.

Our results do not favour any of the two suggested mechanisms. It has been recorded that approximately half (57%) of the plasmodesmata in cells at the AMV infection front are structurally modified, hence, the three-fold increase of plasmodesmata in the same cells can be interpreted either way. Also the plasmodesma-associated compound callose and the antigens recognised by JIM 64 and JIM67 are present in both modified, MP-containing plasmodesmata as well as in unmodified plasmodesmata.

The co-localisation of plasmodesma associated proteins and MP demonstrates that modified plasmodesmata that are actively involved in AMV movement still retained at least some of the biochemical properties. The removal of the desmotubule also means the removal of the proteinaceous particles embedded in or associated with the desmotubule (Overall *et al.,* 1982). This is thought to be the functional part of the plasmodesmata. Our results indicate that, at least part of the plasmamembrane components in the modified plasmodesmata, recognised by the JIM 64 and JIM67 antisera, are still present. The co-localisation of the JIM64/67 antisera and the movement protein of AMV, might be a useful tool to reveal characteristics of the plasmodesmal compounds the antisera recognises.

How plasmodesma formation in existing cell wall takes place still remains to be established, but several models have been proposed. Jones (1976) suggested enzymatic digestion of the cell wall as was supported by Wang *et al.* (1998), who report cellulase activity at the sites of secondary plasmodesmata based on the detection of cellulase reaction products; cuprous oxide in *Lilium davidii.* Lucas *et al.* (1993), suggested the presence of specific recognition molecules at the plasma

membrane-middle lamellar interface that could interact with molecules activating the wall digesting enzymes. The phenomenon of novel plasmodesmata induction in AMV-infected plant cells may provide a good model system for further detailed studies on the dynamics of plasmodesma formation and disintegration.

Acknowledgements

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Chapter 6

Towards identification of host proteins interacting with viral MPs.

Summary

A host protein with affinity to the AMV MP was identified from an expression library of *N. benthamiana* using the two-hybrid system and the MP as bait. This protein, denoted AD3, was expressed in *E.coli* and an antiserum was prepared. Using this antiserum the subcellular location of the protein was established using immunoblotting and immuno-labelling for light and electron microscopy.

A protein of approx. 32 kDa was identified in whole protein extracts from leaves and roots of *N. benthamiana* as well as in extracts from various other plants. Subcellular fractionation of leaf and root samples showed that the protein was predominantly present in the membrane fraction of plant cells. Immunogold labelling demonstrated the presence of the protein in the plasma membrane in both leaf and root tissue. Immuno fluorescent staining of protoplasts showed a similar pattern. However, in AMV-infected tissues and protoplasts, no co-localisation of the AD3 protein and the viral MP could be found. The relevance of these observations is discussed.

Introduction

Plant virus intercellular movement involves both viral components as well as host components. For several viruses the viral components, essential for cell-to-cell movement, have been identified and are referred to as movement proteins (MP) (reviewed by Carrington *et al.,* 1996). These MPs support the movement of the viral genome in form of a virion (e.g. CPMV,) or as 'naked RNA' (e.g. TMV) through plasmodesmata in the plant cell wall. To fulfil its function in either movement mechanism, the MP obviously interacts with components of the host cell. After synthesis the MP is targeted to the periphery of the cell to reach the plasmodesmata which it must modify to allow passage of the large viral genome or virion. For TMV, directly after synthesis the MP associates to the viral RNA and this nucleoprotein complex is targeted to plasmodesmata possibly via the host cell's cytoskeleton (reviewed by Citovsky, 1999). Based on fluorescent co-localisation studies and the use of destabilising drugs, McLean *et al.* (1995) and Heinlein *et al.* (1995) showed that the MP associates with the cytoskeleton, in particular the microtubules, or cytoskeleton binding elements. It is, however, still not established whether MP intracellular translocation takes place directly on the microtubules by mimicking cytoskeleton binding elements or via the endoplasmic reticulum (ER), which itself traffics through the cell via cytoskeleton elements (Heinlein *et al.,* 1998). In this respect Huang and Zhang (1999) found an association of the *Alfalfa mosaic virus* (AMV) MP with the ER and suggested that intracellular trafficking of this MP to the periphery of the cell takes place via the ER, probably as an integral ER membrane protein. Further interactions between the MP and host components, are obvious from the necessity of plasmodesmal modification to allow virus transport. Plasmodesmata are either modified in their permeability for macromolecules (increase in size exclusion limit; e.g. TMV (Wolf *et al.,* 1989) or more drastically, in their structure a.o. assembly of transport tubules in case of CPMV infection (van Lent *et al.,* 1990a).

For AMV we have demonstrated that, at the border of infection, both MP and CP are present in the plasmodesmata and these plasmodesmata are structurally modified (enlarged diameter and absence of the desmotubule) probably to allow assembly of a transport tubule and subsequent transport of the virion (Kasteel *et al.,* 1997 and Chapter 2; Van der Wel *et al.,* 1998 and Chapter 4). These observations indicate that the AMV-MP interacts with components in the plasmamembrane and/or plasmodesma. However, the number of plasmodesmata apparently increases in cells that are located within the expanding infection sites (Chapter 5) suggesting that AMV-infection leads to formation of new plasmodesmata. It is not established whether these new plasmodesmata are involved in AMV transport or whether the plant cell makes them to restore intercellular communication, after existing plasmodesmata have been modified for virus cell-to-cell movement. When assuming that the virus induces new channels for its cell-cell movement, the MP would interact with plant proteins involved in the formation of a novel plasmodesmal pore. In this respect Lucas (1993) already suggested the existence of recognition molecules present in the plasma membrane, which would activate cell wall digesting enzymes. Recently, Dorokhov *et al.* 1999 and Chen *et al.* 2000 reported that a ubiquitous cell wall-associated plant enzyme pectine methylesterase of *Nicotiana tabacum* L. specifically binds to the MP

of TMV and is able to bind RNA. The authors suggested that this enzyme, considered to be responsible for chemical modifications of pectin and involved in cell wall growth and regeneration, could serve as a host cell receptor involved in TMV cell-to-cell movement.

To identify host components that show an affinity for the AMV MP we have employed the yeast two hybrid system (Fields and Song, 1989), using a genomic library of *N. benthamiana* and the AMV MP as bait. The results from this two hybrid screening are extensively described in the thesis of L. Jongejan, Leiden University (Jongejan, 2000). In summary, screening of the genomic library resulted in the identification of 10 different genes whose products repeatedly showed an affinity to the MP. One host protein in particular, a protein with a calculated size of 29.5 kDa, denoted AD3, appeared very promising based on its sequence and the presence of its RNA in tissue. The RNA of AD3 was detected in healthy and AMV-infected *N. benthamiana* and *N. tabacum* cv. Samsun NN leaves using Northern blot analysis. Furthermore, the *ad3* gene showed homology with expressed sequence tags of *Arabidopsis* (60-80% on protein level), a hypothetical protein (28.4 kDa) of *Escherichia coli* (50% on protein level) and a bacterial ferripyochelin binding protein (FBP) from *Pseudomonas aeruginosa* and *Methanococcus jannaschii* (30-55% on sequence and protein level). FBP is a plasma membrane protein that, at low iron levels, binds excreted pyochelin so that iron attached to the pyochelin can be released in the cell. So far, the FBP has not been detected in a plant genome.

The function of the AD3 protein *in planta* is of course unknown. Here we have studied the expression and *in situ* localisation of this protein in uninfected and AMV-infected plant tissues to establish some of the characteristics of this host protein.

Methods

The production of AD3 protein and antisera

The PCR amplified AD3 construct was cloned into a pGEX-2T vector fused to *Schistosoma japonicum* glutathione S-transferase (GST) (Pharmacia) by L. Jongejan. Both a truncated *ad3* gene (missing 261 N-terminal base pairs) and the complete *ad3* gene were cloned into the fusion vector, which was then transformed into *E.coli* strain BL21. The fusion genes were both expressed to high levels and the GST-AD3 fusion protein was purified using the GST purification module (Pharmacia). The AD3 was cleaved from the GST using thrombin and the free GST was removed using the purification module. This resulted in large quantities of truncated AD3 protein and complete AD3 protein, however, the complete AD3 protein aggregated into an insoluble substrate. The truncated AD3 protein was, therefore, used to produce antisera. Antisera were raised in rabbit (by Eurogentec Belgium) and rat (at the *Centrum Kleine Proefdieren*, Wageningen University). Prior to immunisation pre-immune serum was obtained from the animals. The immunisation protocol consisted of three subcutanous injections at intervals of 14 days with truncated AD3 protein (20, 100 and 100 μg respectively) emulsified in Freund's incomplete adjuvant. Samples of blood were taken 28, 66 and 80 (final bleeding) days after the final injection and the

quality of the sera was tested on western blots of *E.coli* fractions with and without GST-AD3 production, before and after IPTG induction of the *gst-ad3* gene.

Western immunoblot analysis

Samples from roots or leaves of *Nicotiana benthamiana* plants were processed for Western blot analysis by homogenising 0.1 g tissue in 0.1 ml PBS. The homogenate was mixed 1:1 with 2x Laemmli loading buffer, boiled for 2 min and centrifuged at 14000 g for 1 min. Ten μl of the samples were applied on a 13.5% SDS-polyacrylamide gel as described hereafter.

Subcellular extracts were made from leaves of *N. benthamiana, N. tabacum, Vigna unguiculata, Hordeum vulgare, Datura stramonium* and *Zea mays* as described by Kormelink *et al.* (1994). In brief, 1 g leaf tissue was powdered in liquid nitrogen, resuspended in 2 ml grinding buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 5 mM dithiothreitol), filtered through two layers of cheesecloth and centrifuged for 10 min at 1,000 g. The pellet, containing the crude cell wall fraction (Pe-1), was washed two times in 2 % (v/v) Triton-X-100 in grinding buffer. The supernatant was again centrifuged for 30 min at 30,000 g to obtain the pellet (Pe-30), containing the membrane fractions, and the S-30 supernatant which contains the cytoplasmic fraction. The protein concentration was calculated using the Bio-Rad Protein Standard and the measured OD_{595} of the samples. The fractions were then mixed with 10% SDS to equalise the protein concentration.

Ten μl of the extracts were applied on a 13.5% SDS-polyacrylamide gel (Leammli *et al.*, 1970). The SDS-polyacrylamide gel was transferred to Immobilon membrane by electroblotting for 1 hr at 0.08 Amps/25 V (Biorad semidry transfer cell). Membranes were blocked in 5% (w/v) dried milk (ELK) in PBS for 30 min, briefly washed in PBS and incubated in anti-AD3 serum or pre-immune serum diluted in PBS containing 0.05% (v/v) Tween-20 for 30 min. After washing three times 10 min in PBS-Tween the membrane was incubated with goat anti-rat (or anti-rabbit) immunoglobulins conjugated to alkaline phosphatase and diluted in PBS-Tween (1:3000). The blots were stained using nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP) as substrate.

Electron microscopy

Samples of *N. benthamiana* leaf tissue, healthy or infected with AMV, and root tips from healthy plants were fixed in 2% (w/v) paraformaldehyde and 3% (w/v) glutaraldehyde in phosphate/citrate buffer (0.1 M $Na_2HPO_4.2H_2O$, 2.7 mM citric acid and 1.5 mM $CaCl_2$), pH 7.2 for 24 hrs at 4°C. The tissues were then dehydrated and embedded in LR Gold as described by Van Lent *et al.* (1990b). Alternatively, after fixation, leaf samples and root tissues were subjected to freeze-substitution or prepared for cryo-sectioning as described by van der Wel *et al.* (1998).

Immuno-gold labelling using the antisera raised against the AD3 protein was performed using the appropriate conjugate, protein-A gold (10 nm) for the antiserum from rabbit and goat anti-rat IgG conjugated to 10 nm gold for the antiserum from rat, using the procedure described by van der Wel *et al.* (1998).

Fluorescent microscopy

Protoplasts were isolated from healthy and AMV-infected *N. benthamiana* leaves as described by Van Dun *et al.* (1988). The protoplasts were fixed either directly after isolation or after an incubation period of 16 h at 25 °C. Fixation was performed for 20 min using ethanol, acetone, or 1% (w/v) paraformaldehyde in PC-buffer and, in case of fixation with paraformaldehyde, followed by de-staining with ethanol for 20 min to remove auto-fluorescence of the chloroplasts. Immunofluorescent staining using anti-AMV MP, anti-AMV CP or anti-AD3 was performed according to Kasteel *et al.* (1997). The AD3 sera were used in different dilutions and with the appropriate secondary antibody conjugated to FITC. The serum raised in rats and diluted 1:1000 appeared to give the best results.

Results

Expression of AD3 and specificity of the antisera

The complete AD3 protein and the truncated AD3 (tr-AD3) fused to GST were successfully expressed in *E.coli*. However, the AD3-GST fusion protein appeared to be insoluble and only the tr-AD3-GST fusion protein of approx. 46.5 kDa was obtained in a soluble form (Figure 1). The fusion product was cleaved into tr-AD3 and
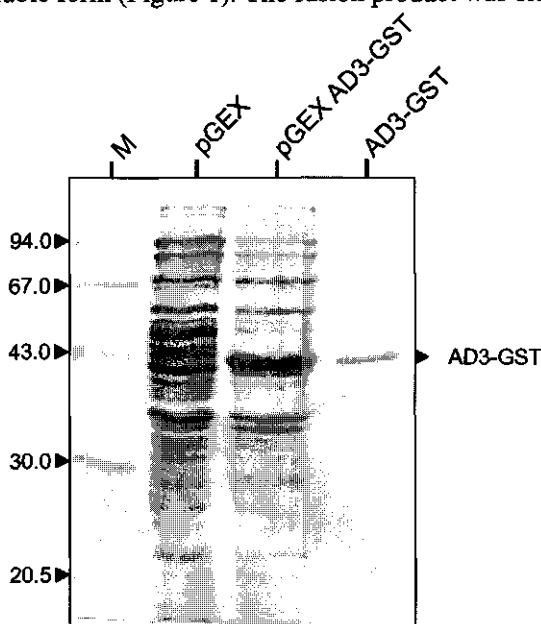
Figure 1
Production and purification of fusion protein tr-AD3-GST (C-terminal part of AD3 fused to glutathione S-transferase (46.5 kDa). In first lane, low molecular weight marker (M). The E.coli suspension before induction of the pGEX vector in the second lane (pGEX) and after induction of the tr-AD3-GST protein in the third lane (pGEX AD3-GST) The purified AD3-GST after processing the GST purification module (AD3-GST). E.coli samples and the fusion protein were resolved on a SDS polyacrylamide gel and stained with Coomassie brilliant blue.

GST using thrombin and subsequently the GST was removed, leaving a suspension of pure tr-AD3 protein. This purified tr-AD3 was used to immunise a rat and a rabbit.

The sera obtained from different bleedings of the rabbit did not differ much in quality and all reacted to the tr-AD3-GST fusion protein in immunoblots of IPTG induced *E.coli* fractions. The antiserum raised in rat also reacted to the tr-AD3-GST fusion protein in IPTG induced *E.coli* fractions. In the coomassie brilliant blue stained gel of purified protein fractions it became apparent that the AD3 protein is unstable and dissociates into smaller subunits which are recognised by the antiserum using Western blot analysis.
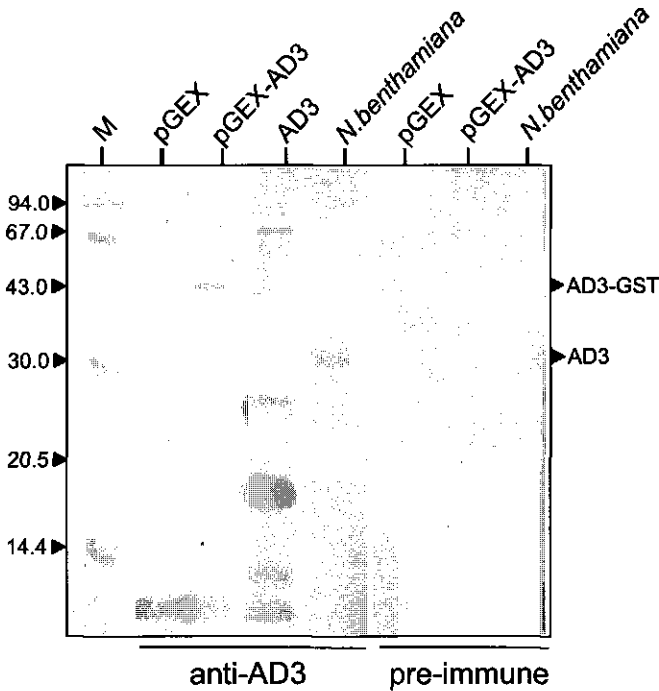
Figure 2

AD3 antiserum raised in rat (diluted 1:2000) detected the AD3 protein on western blots of fractions of E.coli expressing pGEX AD3-GST and in leaf tissue. Low molecular weight marker (M) is shown in the left lane. The control fraction of E.coli *cells not expressing the pGEX AD3-GST demonstrates some background in lane 2 (pGEX). The* E. coli *cells induced for the expression of the fusion protein (46.5 kDa) shows a clear additional band at the expected size and an extra band at approx. 35 kDa (pGEX-AD3). The purified AD3 protein (dissociated in 2 smaller subunits) is detected by the serum in the fourth lane (AD3). In N.* bentamiana *a protein with the expected size (approx. 30 kDa) was detected (*N. benthamiana*). The pre-immune serum does not recognise the AD3-GST fusion product nor the protein in N.* benthamiana.

In *N. benthamiana* fractions, both sera reacted to a 32 kDa protein band which is approximately the expected size of AD3 (29.5 kDa). Here the serum from rabbit and rat reacted differently. Besides a clear band at 32 kDa the serum from rabbit also showed reaction to several other proteins in the plant fractions, while the preimmune serum showed no staining at all. The serum from rat more specifically reacted to the 32 kDa protein (Figure 2), expected to be the AD3 protein. The antiserum raised in rat, therefore, appears to be more specific and was used in most experiments.

Location of the AD3 protein

In total root and leaf samples of *N. benthamiana* the AD3 protein was detected by immunoblotting using the antiserum from rat. To determine more specifically the location of AD3 in plant tissues, subcellular fractions were made from *N. benthamiana* leaves and root. A 32 kDa protein was specifically detected in the membrane enriched fraction denoted Pe-30 (Figure 3), which was expected to be the AD3 protein (calculated size of 29.5 kDa). This protein appeared to be absent from the cytoplasmic fractions (S-30) and the cell wall enriched fractions, containing a.o. plasmodesmal compounds (Pe-1).
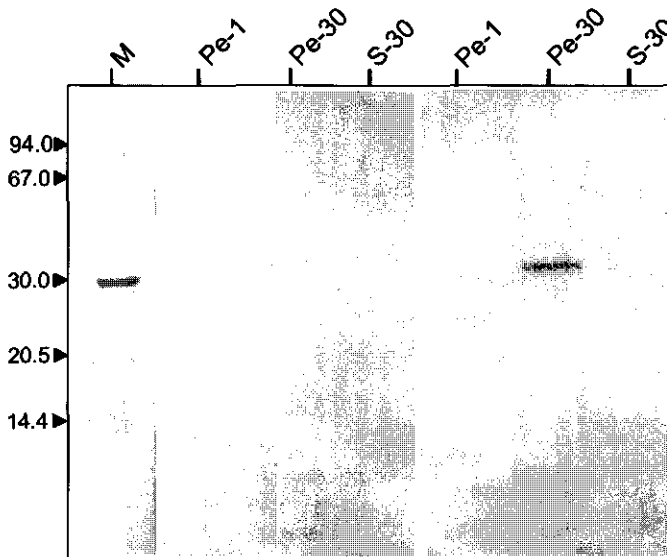
Figure 3
Western blot detection of AD3 using rat antiserum (1:2000) in subcellular fractions of N. benthamiana, root (lanes 2-4) and leaf tissue (lanes 5-7). Both the cell wall and cytoplasmic enriched fractions (respectively Pe-1 and S-30) do not contain detectable amounts of AD3 whereas in the membrane fraction (Pe-30) AD3 was detectable. Low molecular weight marker (M) is shown in lane 1.

In subcellular fractions of different plant species from different families, the antiserum reacted with a similar sized protein also exclusively present in the membrane-enriched fractions (Figure 4; data from other subcellular fractions not shown). The plant species

N. benthamiana, N. tabacum, V. unguiculata were selected for their susceptibility for AMV-infection, while *D. stramonium* is not susceptible for AMV. *H. vulgare* and *Z. mays* were included as representatives for monocotyledon species. In the membrane fractions of *N. tabacum* and *Datura stramonium* a relatively high amount of protein was detected, whereas the signal in *Vigna unguiculata, Hordeum vulgare,* and *Zea mays* was less abundant.To establish whether the AD3 protein content was different in cells infected with AMV, leaves with a progressing front of AMV-infection were analysed for the presence of AD3. Samples were made from the three different zones of infection, respectively uninfected, border of infection and fully infected (see also chapter 4). The AD3 protein could be found predominantly in membrane fractions from all zones with no conclusive differences in AD3 protein content.
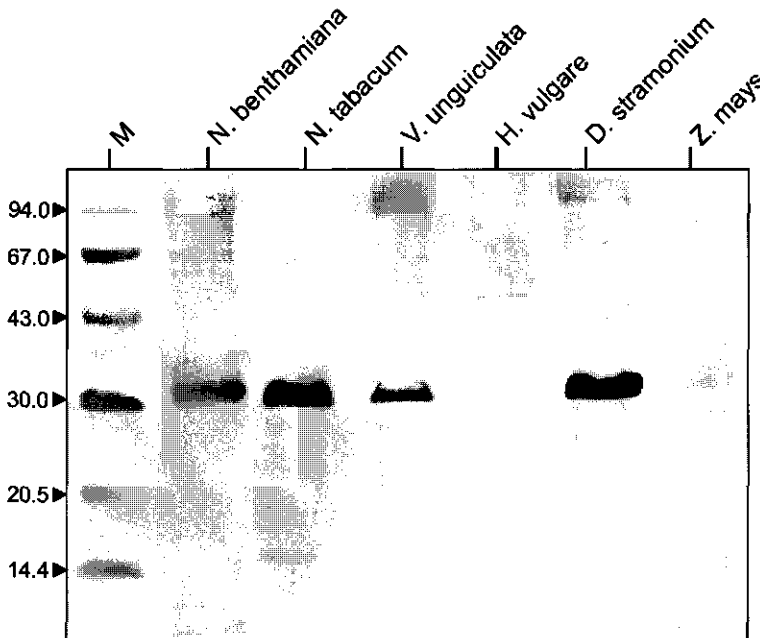
Figure 4
Detection of AD3 on western blots of subcellular membrane enriched fractions of leaf tissue from different plant species. Antiserum was raised in rat and diluted 1:2000, the marker lane (M) contains low molecular weight marker.

Subcellular localisation of AD3
To determine the subcellular location of the AD3 protein in *N. benthamiana* cells, protoplasts were analysed using immuno-fluorescent staining by anti-AD3 serum from rabbit. Different fixation protocols were tested. Only a fixation of the protoplasts in acetone resulted in peripheral fluorescent staining showing some concentrated spots, which were not observed when using the pre-immune serum (Figure 5). In AMV infected protoplasts, showing tubular structures at their surface (see Chapter 2), AD3 localisation was similar to that in uninfected protoplasts. No co-localisation between AD3 and the MP could be observed.
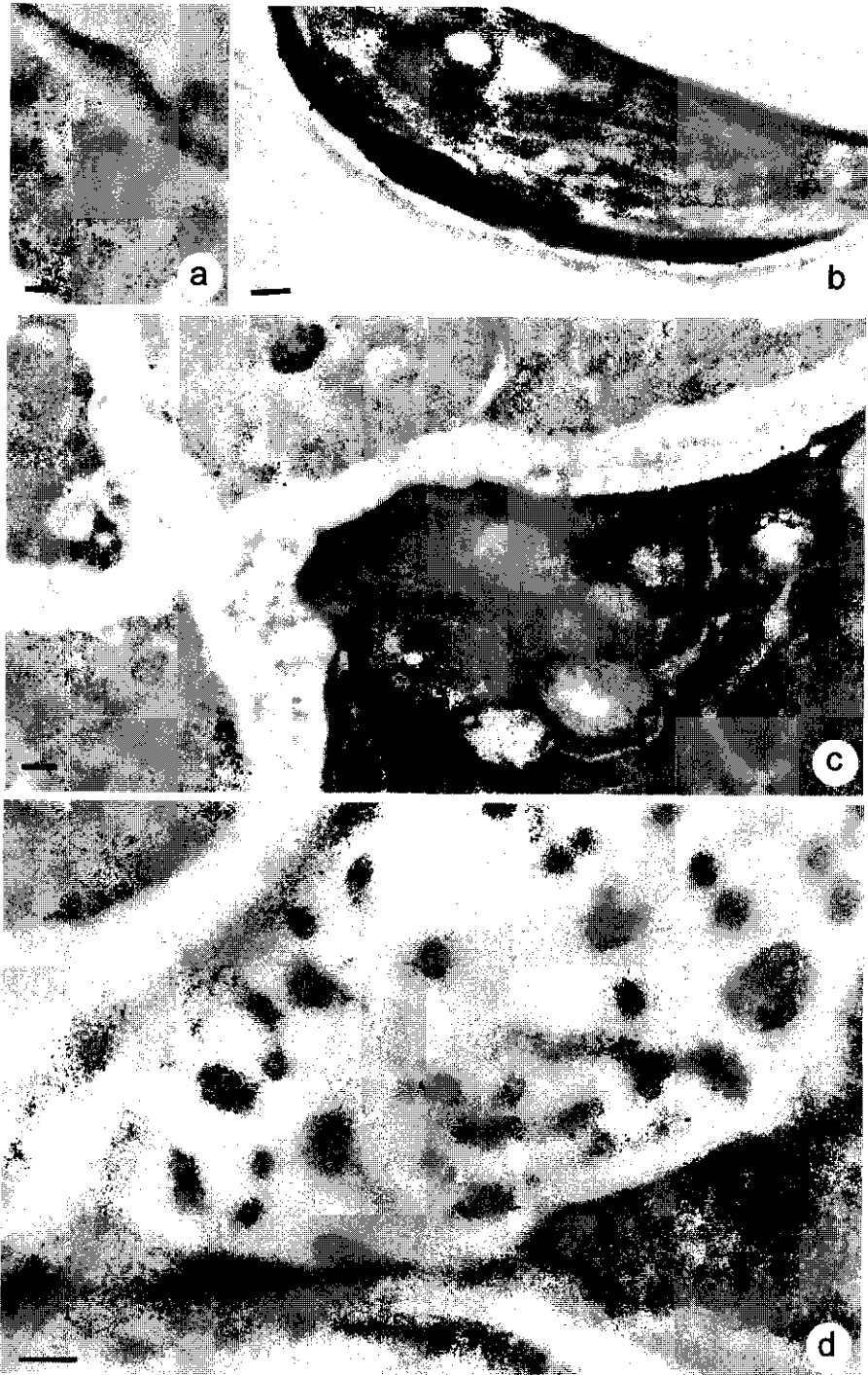
Figure 6
Imunogold labeling of AD3 in LRG embedded leaf tissue (a,b) and root tip tissue (c,d) of N. benthamiana.Bar represents 10 nm

To establish a more specific subcellular location of the AD3 protein, sections of N. benthamiana leaf tissue were treated with anti-AD3 from rat and subsequently labelled with goat-anti-rat conjugated to gold particles. Specific gold labelling was detected on the plasma membrane of mesophyll cells (Figure 6 a,b). However, as expected, the protein was not abundantly present and therefore difficult to detect in most cells. In leaf tissue containing the AMV infection border no difference in AD3 localisation was observed. In particular, no specific labelling of modified plasmodesmata, the location of the AMV MP (see Chapter 4), could be found.

Also in root-tip tissue, immuno-gold labelling of the AD3 protein was found, particularly at the plasma membrane (Figure 6c) and frequently on lomasomes or paramural bodies connected to the cell wall (Figure 6d). The gold label was mostly observed on the membranes of the cytoplasmic vesicles of these organelles. Thus also in the lomasomes, the membrane is labelled.

Discussion

In search for host proteins that show an affinity to the AMV-MP and thus may play a role in the mechanism of AMV cell-to-cell movement, the yeast two-hybrid system was employed to screen an expression library of *N. benthamiana* using AMV MP as bait. Several host proteins with different affinities for the MP were identified, one of which was the so-called AD3 protein (Jongejan, 2000).

A truncated form of the *ad3* gene was successfully expressed in *E. coli* and purified to produce antisera and these antisera reacted specifically to *E. coli*-expressed AD3 proteins. Furthermore, these antisera identified a protein of approximately 32 kDa in whole protein extracts from *N. benthamiana*, assumed to be the AD3 protein, which has a calculated size of 29.5 kDa.

Besides *N. benthamiana*, the AD3 proteins were also found in different concentrations in *N. tabacum, Datura stramonium, Vigna unguiculata, Hordeum vulgare,* and *Zea mays.* This indicates that the protein is conserved amongst different plant families and between Dicotyledons and Monocotyledons, although the latter seem to contain lower quantities of the protein. Also, there is no clear correlation between presence of AD3 and susceptibility to AMV, as *D. stramonium* is unsusceptible for AMV infection and no differences in AD3 content could be observed when compared to the susceptible plants.

As AD3 was found on the basis of its affinity for the AMV MP, it was anticipated that its subcellular location would be related to AMV movement and AMV MP. However, no evidence has been found that supports these expectations. The presence of the AD3 predominantly in the membrane fractions of different plant species demonstrates a preserved location and suggests a conserved function. Since the AD3 protein was not detected in cell wall fractions, the protein is apparently not part of the plasmodesmal structure, as plasmodesmata are preserved in this fractions, which was confirmed by electron microscopical analysis (data not shown).

The presence of AD3 in membranes, in particular the plasma membrane and the absence from plasmodesmata was confirmed by *in situ* immunogold localisation. In root tissue AD3 was detected in lomasomes and plasma membranes. Lomasomes or

paramural bodies are the storage sites for materials involved in cell wall metabolism and both saccharide residues and cellulase have been detected in the vesicular structures embedded in cell wall appositions (Chamberland *et al.*, 1989, Baird and Reid, 1992). In root tip tissue, numerous lomasomes are present and needed for the constant production of new cell wall material for dividing and elongating cells. The presence of AD3 in the lomasomes combined with the function of these organelles, makes it is tempting to speculate that the AD3 protein has a role in the construction or the decline of the plant cell wall. In leaf tissue only cell elongation took place and obviously less lomasomes were present. The AD3 protein, here detected only at the plasma membrane, could have a similar function in cell wall construction or decline. This would be very important, not only in the cell life cycle, but also in the process of cell-cell movement of plant viruses. Considering that AMV needs to modify the diameter of the plasmodesmal pore or to induce a novel channel in the cell wall to establish its transport, cell wall reconstruction is an essential feature of the AMV movement mechanism. The recent identification of the cell wall residing enzyme pectin methylesterase, active in chemical modifications of pectin and thought to be involved in cell wall growth and regeneration, as a possible receptor for TMV-MP (Dorokhov *et al.*, 1999), but also for the MP of other plant viruses (Chen *et al.*, 2000) is in this respect very interesting.

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Chapter 7

General discussion

The research described in this thesis, was part of an ALW-sponsored program, involving three PhD projects, and which focused on the interaction of viral movement proteins (MPs) with plasmodesmata and host proteins. One of the approaches was to identify MP-binding host proteins using the MPs of two viruses, with a very different mechanism of cell to cell movement, as two distinct probes. For this, *Cowpea mosaic virus* (CPMV) and *Alfalfa mosaic virus* (AMV) were selected, as previous work on CPMV had demonstrated that this is a representative of those plant viruses which use tubule-guided movement of whole virions as transport mechanism, while in view of its genetic relationship to *Tobacco mosaic virus* (TMV), AMV was expected to use the tobamo-like movement mechanism, i.e. movement of the viral genome in a non-virion form. Thus it was anticipated that the MPs of these two viruses would interact in a distinct way to the host's plasmodesmata and host proteins.

The aim of the cytological line of the program, as summarised in this thesis, was to study the behaviour of the viral proteins involved in the movement process, and their interaction with host proteins. The outcome of these studies, the implications for the concept of virus transport and new questions to be addressed, are discussed here below.

The mechanism of AMV-movement

At the onset of this thesis research, the strategy of AMV intercellular movement was not without controversy. The capacities of the MP to bind RNA (Schoumacher *et al.*, 1992) and to increase the plasmodesma size exclusion limit (SEL) (Poirson *et al.*, 1993) were typical for tobamo-like movement of a RNA-MP complex, a mechanism in which the CP has no role (Carrington *et al.*, 1996). However, van der Vossen *et al.* (1994) showed by mutational analysis that both the MP and CP of AMV were essential for systemic movement of AMV and in this aspect AMV clearly differed from TMV. The essential role for CP in AMV movement was explained by our observation by electron microscopy (Chapter 2; Kasteel *et al.*, 1997a) that AMV induces MP-containing tubular structures as projections from the protoplast surface and that these tubules contain virions. This in striking similarity with CPMV, a virus which employs a tubule-guided movement mechanism (Wellink and van Kammen, 1989; van Lent *et al.*, 1991). Like with AMV we showed that also *Brome mosaic virus* (BMV), another virus species within the *Bromoviridae* family, was capable of forming MP-containing tubules with virions (Chapter 2, Kasteel *et al.*, 1997a). However, it seems that there is no single unambiguous movement mechanism employed by all viruses within the *Bromoviridae*, but cell-to-cell viral movement appears to be more complex within this family as not all viruses require the CP for movement. *Cucumber mosaic virus* (CMV; Canto *et al.*, 1997), AMV (van der Vossen *et al.*, 1994; Kasteel *et al.*, 1997a) and BMV (Schmitz and Rao, 1996) require the MP and CP to move from cell to cell and the MP is able to aggregate into tubular structures, whereas the CP was found dispensable for movement of *Cowpea chlorotic mottle virus* (CCMV; Rao, 1997), a virus closely related to BMV, and for which no tubular structure formation is reported. An important observation was recently made by Canto and Palukaitis (1999). A CMV-MP mutant, unable to form tubules in protoplasts, was able to move systemically in tobacco plants but, unlike wildtype virus, was not able to traffic through plasmodesmata interconnecting tobacco epidermal cells. The authors therefore suggested that CMV could employ different, tissue-depending, mechanisms of intercellular movement. This could also be the case for AMV.

During our studies of AMV MP- and CP-mutants (Chapter 3) that are restricted in their cell to cell movement, we made an interesting observation on the behaviour of these mutants in plant tissue. Although, in mesophyll protoplasts these mutants were either unable to produce stable virions (CP4P and CPN199) or tubular structures (SP6 and SP7) and in leaves infection of these mutants was restricted to single epidermal cells, some spread of infection with these mutants was observed in cortex cells surrounding the leaf veins. Like suggested for CMV, our observations point to the possibility that AMV is not limited to a single mechanism of movement, but the mechanism employed may be tissue-dependent. However, to conclusively explain the tissue dependent spread observed with these mutants, further research is needed. Cortex cells will differ from mesophyll cells in various aspects. There may be differences in cytoplasmic environment which could result in stabilisation of otherwise unstable mutant viral proteins or, alternatively, there may be differences in plasmodesmal structure, in particular SEL which could allow for transport of another form of infectious complex. It has been established by now that differences in plasmodesmal SEL exist between cells from different tissues (Kempers *et al.*, 1993; Kempers and Bel, 1997; Imlau *et al.*, 1999) or even within one type of tissue in different developmental stages (Oparka *et al.*, 1999). In this respect, non-invasive detection techniques using the gene for green fluorescent protein (GFP) as a fluorescent marker in viral constructs could make it possible to study tissue specific properties like the permeability of the plasmodesmata in cortex tissues, as has recently been demonstrated by Oparka *et al.* (1999).

Tubule-guided movement of AMV through modified plasmodesmata

The inability of the above mentioned AMV mutants to move from cell to cell in mesophyll tissue and this phenotype in mesophyll protoplasts (no tubules with mutants SP7 and SP6 and no stable virions with mutants CP4P and CPN199) sustains a major role of the tubule-guided movement mechanism in AMV systemic spread. If that is the case, in analogy to e.g. CPMV, virion-containing tubules should be present in plasmodesmata interconnecting AMV-infected cells. Only one report existed on the observation of structures that could resemble tubules (Godefroy-Colburn *et al.*, 1990) and also during our research we have been able to find such structures, that were best preserved after cryo-fixation and cryo-sectioning of AMV-infected leaf tissue. In this AMV differs from CPMV, as with the latter frequently long virion-filled tubular structures are observed in plasmodesmata and extending into the cytoplasm. The difference between AMV and CPMV could first of all be explained by the very different translation strategy employed by these viruses. Whereas with CPMV the MP and CPs are translated in equimolar amounts from RNA2 during infection resulting in continuous growth of the tubule even after early and successful movement of virions to the neighbouring cell, the AMV-MP is translated from a subgenomic messenger RNA. The latter strategy could account for the temporal presence of this MP in infected cells (Godefroy-Colburn *et al.*, 1986; Stussi-Garaud *et al.*, 1987). Our *in situ* analysis of the presence of MP in AMV-infected plant tissues, confirms the transient nature of MP in infected leaf tissue. By immunogold labeling we have shown that the MP, but also the CP, were only present in plasmodesmata of *N. benthamiana* cells within a narrow zone of 3-4 cells at the progressing infection front (Chapter 4). Neither protein was present in plasmodesmata of cells that were behind this zone and 'fully' infected with AMV. Once virus has moved from an infected cell to a healthy

neighbouring cell, there is no need anymore for a functional MP. Hence, the transient presence of MP at the infection border could be considered efficient and may result in the formation of tubules restricted to the plasmodesmal pore. The limited number of cells in "movement mode" and the limited and temporal production of the MP may explain the difficulties encountered in observing the transport tubules *in planta*.

Other remarkable phenotypic features of AMV movement comprise the structural alteration of plasmodesmata, in particular the increase in plasmodesmal pore, that coincided with the presence of MP and CP and the apparent increase in number of plasmodesmata, all observed only in cells at the infection front. Both observations support the idea that plasmodesmata are dynamic structures that can be made or altered during development and under influence of physiological or metabolical changes (Crawford and Zambryski, 1999). However, the mechanism by which plasmodesmata are formed in secondary cell walls is not at all understood (Lucas *et al.*, 1993, Wang *et al.*, 1998).

We have not been able to establish whether AMV moves through newly formed pores or whether it uses existing plasmodesmata after modifying them. In any case the virally modified plasmodesmata contained callose and two unidentified plasmodesma-associated proteins described by Waigmann *et al.* (1997) and which are recognised by antibodies JIM64 and JIM 67 (Chapter 5). The presence of these components in pores that are implicated in AMV movement, suggests their plasmodesmal identity.

The increase in number of plasmodesmata could be essential for the cell to sustain its intercellular communication. As virus generally spreads rapidly over long distances through the phloem from metabolic source tissues to sink tissues, young (sink) leaves become efficiently infected. In our studies we have analysed the infection border present in young developing leaves of *N. benthamiana*. Such leaves, at a certain moment, undergo a transition from sink to source (from leaf top to base). Oparka *et al.* (1999) showed that such a transition involves a drastic change in plasmodesmal conductivity (SEL) and structure (from linear to branched). Viruses may benefit from or influence such existing and activated host mechanisms, that regulate macromolecular transport through plasmodesmata, to support their own cell-to-cell movement. To establish whether AMV is capable of triggering such a mechanism and evoke its own "transport plasmodesma" when necessary, studies should be carried out in fully developed source leaves or in cells that are symplastically isolated like the guard cells of mature stomata.

AMV MP interaction with host proteins
Either way, AMV seems to be able to interact with existing host specific mechanisms that are involved in wall or plasmodesmal regulation. This view is supported by recent independent reports of Dorokhov *et al.* (1999) and Chen *et al.* (2000) who showed an interaction between TMV MP (but also *Cauliflower mosaic virus* MP, a virus employing a tubule-guided movement mechanism!) and a ubiquitous cell wall-associated plant enzyme, pectine methylesterase. This enzyme is involved in cell wall turnover, affects cell wall porosity [(Chen *et al.*, 2000) and references herein] and has been implicated in more specialised cellular processes such as plant response to pathogen attack (Markovic and Jornvall, 1986). The enzyme was identified by a renatured blot overlay assay using

TMV-MP and cell wall proteins and MP binding was confirmed in the yeast two-hybrid system.

In this collaborating research program our partner from Leiden University has employed the yeast two-hybrid system to identify host proteins that interact with the AMV MP. Unlike the biochemical and 'cell wall directed' approach of Dorokhov *et al,* (1999) and Chen *et al.* (2000), our approach to screen a cDNA bank from *N. benthamiana* could lead to identification of additional host components that are otherwise associated with the MP. Apart from interactions with cell wall and plasmodesmal components, it is expected that viral MPs will interact and show affinity with a number of host components that are related to intracellular targeting and trafficking (e.g. the endoplasmic reticulum and cytoskeleton) as has been suggested for AMV by Huang and Zhang (1998) and has clearly been shown for TMV (Heinlein *et al.,* 1995; McLean *et al.,* 1995). So far the yeast two-hybrid analysis has revealed one interesting host protein, denoted AD3, with a clear affinity for the AMV-MP. Sequence analysis revealed that AD3 has no clear membrane spanning domains and shows homology with expressed sequence tags of *Arabidopsis* and with the ferripyochelin binding protein (FBP), a plasma membrane protein from *Pseudomonas aeruginosa* and *Methanococcus jannaschii* (Jongejan, 2000).

Using antisera against the AD3 protein, Western blotting as well as *in situ* immunofluorescent and immunogold staining confirmed the presence of this protein in membrane fractions in roots and leaves of *N. benthamiana.* In leaves AD3 was present in the plasma membrane and in roots the proteins was also found in so-called lomasomes. These lomasomes (or paramural bodies) are invaginations of the plasma membrane in the cell wall and are thought to be involved in cell wall formation (Chamberland *et al.,* 1989; Baird and Reid, 1992). The presence of AD3 in such lomasomes suggests a role in this process. AD3 thus may play a role in the mechanism of AMV-induced modification of the cell wall or of the plasmodesmata.

To gain more insight in the functioning of the AD3 protein and its effect on AMV infection, transgenic plants expressing the *ad3* gene in sense and anti-sense orientation were constructed. At present only very preliminary results are available. Individual transformed plants contained very different levels of AD3 protein. Two plants containing a relatively high amount of AD3 both died prematurely, indicating that a high level of AD3 may reduce the vitality of these plants. First inoculation experiments of plants from the S1 progeny containing the AD3-gene in sense and anti-sense orientation, indicate that a number of plant lines may be resistant to AMV infection. However, as experiments were not repeated no conclusions can yet be drawn.

Suggestions for further research

The results described in this thesis demonstrate that AMV employs, in parenchyma tissues, a tubule-guided mechanism for its cell-cell movement (Figure 1). In contrast to the so far described tubular structures of other viruses, the AMV tubules appear to be short and restricted to the plasmodesmatal pore. Furthermore, our results indicate that at the expanding infection front, novel pores penetrating the cell wall are induced. This observation opens a new view on how viral proteins interact with the host and could elucidate the resistance of older plants, to viruses since plants in later developmental stages lose their ability to form novel plasmodesmata. Understanding these processes might lead to the development of new procedures to combat virus infections in crops. However, the study of plasmodesmal induction is difficult to

Figure 1
Model for the cell to cell movement of AMV. A short MP tubular structure is inserted into a plasmodesmatal pore. Immunological studies revealed that the modified plasmodesmata still contain compounds typically present in plasmodesmata.

tubular
structure

virus
particle
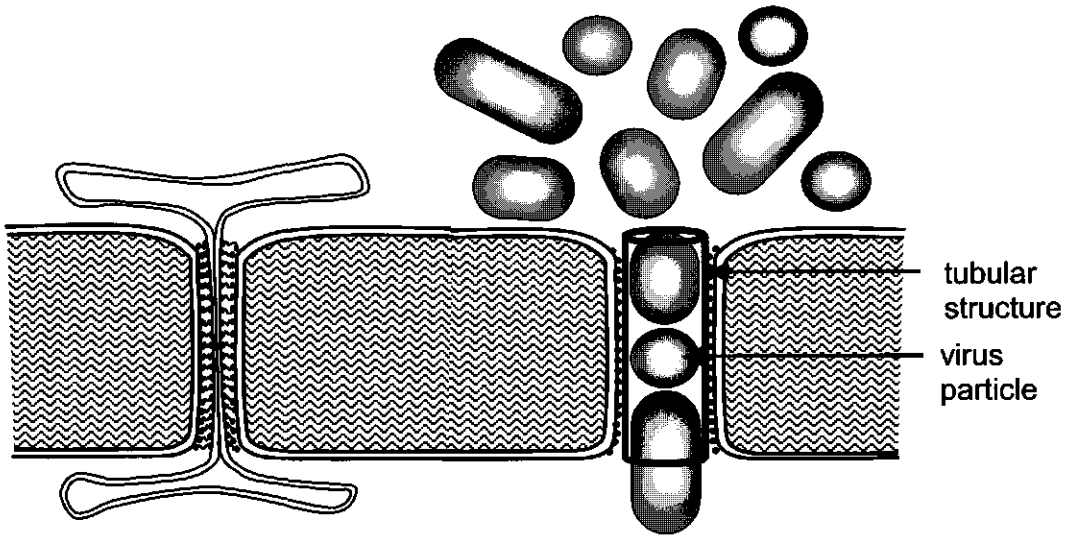
perform and the cellular processes taking place are largely indeterminate and need further research for which some suggestions are done here.

Interactions between viral proteins and host proteins or organelles have been studied in the past and also in this PhD research, but further research in this direction will reveal new insights. For example, the endoplasmatic reticulum (ER) could be used as a target organelle since it has an important role in plasmodesmata formation (Kollmanna and Glockmann, 1991) and in early processes of virus infection, when the MP is transported to the cell periphery (Huang and Zhang, 1998). The trafficking via the ER concentrates the MP to the site where it will function possibly allowing it, in an unknown manner, to construct into the tubule. It is possible to separate the ER membranes from other cell components and these fractions could be used for immuno-precipitation studies, in a similar manner Dorokhov *et al.* (1999) and Chen *et al.* (2000) identified the cell wall associated protein, interacting with the TMV MP.

This cell wall associated protein, identified as a pectin methylesterase, is suggested to play a role in the cell wall porosity. Based on the localisation of the AD3 protein we speculate that this AMV MP interacting protein could also play a role in cell wall modifications. Comparison of the two proteins might reveal more similarities for

example in protein structure or sequence homology. Also, the role of the two proteins in cell wall modifications should be further investigated since this property might result in the allowance of induction of novel plasmodesmata. A process as stated above, presumably employed by AMV.

To further study the potential role of the AMV MP binding protein AD3 in viral movement the intercellular trafficking of this host protein could be studied by transient expression of this gene fused to GFP, and using a CaMV 35s promotor-driven construct, in protoplasts. This could lead to better localisation of the protein in uninfected plant cells as well as to determine its expected co-localisation with the MP in infected cells. The stage in which the AD3 protein would co-localise with the viral protein could then give a clue with respect to its movement supporting function. Alternatively, the *ad3* gene could be transgenically expressed, to define the effects after both co-suppression (no AD3 available) as well as after over-expression on the viral infection, in casu the movement process. We have performed some preliminary experiments in this direction, i.e. transformed *N. benthamiana* with the AD3 cDNA and obtained a few dozen transformed lines. Western blot analyses demonstrate that some lines may over-express AD3 and others possibly are silenced for this gene. AMV inoculation experiments have been done on these first generation lines but do not yet allow any firm conclusions despite the fact that some lines appear to be virus resistant.

The interaction of the MP with plasmodesmata is the stage of infection which could be useful for the detection of plasmodesmal components. Still, hitherto the identification of genuine plasmodesmal components has not been successful. This may be due to the complex structure of the pores, which is not destroyed during cell wall isolation (Chapter 5). To circumvent the difficulty of using complex plasmodesmata, a protoplast system could be used. The MP of several viruses is able to form tubules on the cells in culture and in case of AMV these tubules are even longer than those detected in tissue, allowing higher quantities MP to be examined. There are still remnants of cell wall and plasmodesmata on protoplasts (Ehlers *et al.*, 1996) but functional plasmodesmata are obviously absent. However, the tubules are engulfed by plasma membrane, a membrane that might contain a number of interesting proteins. First studies using immuno-fluorescence, demonstrated the presence of plasmodesmal components recognised by the JIM 64 and 67 antisera, are also present in the MP containing plasmodesmata (data not shown). Further studies might reveal if other known (plasmodesmal) proteins are present in the membrane enveloping the tubule. These proteins could then be further tested for their capability to interact with the MP.

Furthermore, the tubule isolation protocols as described by Kasteel *et al.* (1997b) might also be used to detect other unknown host components present around the tubules and to analyse these by micro-sequencing.

The reporter protein GFP and its mutants have become very useful tools for the analysis of biological processes in living cells. Clearly, this reporter gene can be used in further research. The AMV CP detection method using NBT and BCIP in inoculated leaf ribbons (Chapter 3), could be replaced by GFP expressing virus allowing monitoring of spread in living leaves in time.

Furthermore, GFP will be a very helpful tool to monitor the infection of (existing or new) mutants not only in infected protoplasts and inoculated leaves but also to address the question whether the mutants can spread systemically. Viable AMV-GFP constructs recently have been constructed by Sánchez-Navarro and Bol (personal com.) and will be used to study the movement of (mutant) virus through leaves or protoplasts.

The observed local spread of the AMV mutants (Chapter 3) might be a good starting point for further investigations on the long distance movement of AMV. The results of our research support second tissue dependent mechanism for cell-to-cell movement in cortex tissue, which could represent the long distance movement mechanism of AMV. The mechanism of the movement process taking place in this tissue, is still unknown and needs more research. The RNA binding capacity of the MP could be implicated in these studies as this could well be the basis for the long distance movements of AMV. The distribution of mutants abrogated in the RNA binding capacity of the MP (positioned on the N-terminus of the gene (Schoumacher *et al.*, 1994) could be analysed as well in various tissues of the host plant.

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Summary

For a full infection of a host, plant viruses should be able to multiply in the initially infected cell and to spread to neighbouring cells as to eventually invade the entire plant. The viral transport pathway can in principle be divided into two steps, i.e. cell-to-cell movement within tissues, and long-distance transport via the vascular system. The first process, movement from cell-to-cell, is an "active" process, in terms of that a viral gene product, the so-called "movement protein" (MP) is essentially involved by modifying the host's plasmodesmata to allow viral infectivity to pass. So far at least two different mechanisms of cell-to-cell movement have been described, i.e. the "tobamovirus-type" mechanism, involving transport of virus in a non-virion form through (cytologically) slightly modified plasmodesmata, and the "comovirus-type" mechanism, involving the formation of transport tubules in heavily modified plasmodesmata and allowing mature virus particles to pass. In both mechanisms the viral MP plays a role in the modification of the host's plasmodesmata, and in case of the tobamovirus movement, an increased size exclusion limit of the plasmodesmata is detected.

The PhD research project described in this thesis was part of a programme aiming to unravel the role of the plant's plasmodesmata in virus transport and in cell-cell communication. An important approach in this programme was to use the MPs of two different viruses, each representing both distinct viral transport mechanisms, as probes to identify which host proteins are involved in viral movement and possibly investigate the molecular make up of the plasmodesma. For this purpose the viruses *Alfalfa mosaic virus* (AMV) and *Cowpea mosaic virus* (CPMV) were selected, which moreover share a common host, i.e. *Nicotiana benthamiana*, allowing us to study viral MP-plasmodesma interactions in a single host. Previous work had demonstrated that CPMV uses a tubule-guided movement mechanism, whereas it was anticipated that AMV, in view of its genetic relationship to tomaboviruses, would use the tobamo-type transport. The rational behind the choice for this combination was that the MPs of these two viruses would interact with plasmodesmal constituents or proteins in a distinct way and thus would represent different probes for identifying plasmodesmal proteins and other MP-interacting host proteins in e.g. the yeast two-hybrid system.

This thesis covers the cytological studies within the research programme. The first part of this thesis (Chapters 2-5) deals with the unravelling of the cell-to-cell movement mechanism as utilised by AMV, and in the last part (Chapter 6) cytological studies are presented focussing on a host protein (denoted AD3) interacting with the AMV MP in the yeast two-hybrid system (PhD thesis L. Jongejan, UL, The Netherlands, in preparation).

At the onset of this PhD research hardly any information on the cell-to-cell movement mechanism of AMV was available. Using cowpea protoplasts as test system, it was first shown (Chapter 2) that AMV, like CPMV, is able to form tubular structures on infected plant cells, unexpectedly indicating that AMV would also use a tubule-guided movement mechanism like CPMV. Further analysis indeed demonstrated that these MP-constructed protrusions contain mature virus particles.

To confirm whether AMV, similar to CPMV, would use these structures for a tubule-guided cell-to-cell mechanism, different mutants, defective in systemic spread, were tested for their (in)capability to induce virus-filled tubules. The CP mutant CP4P was previously reported to be unable to form stable virions and was now shown to induce

empty tubules on protoplasts (Chapter 3). Moreover, both MP mutants SP6 and SP7 appeared unable to form tubules on infected protoplasts. Hence, the results with all three mutants indicated that the inability to produce virion-filled tubules on single plant protoplasts coincides with a transport-deficient phenotype. These three mutants were completely deficient in cell-cell movement in parenchyma tissues, a restricted spread of these viruses to neighbouring cells was observed in cortex cells surrounding the vascular system. Whether this indicated a second, tissue-dependent minor movement mechanism of AMV or whether this spread reflected distinct properties of plasmodesmata of cortex cells, was not further investigated.

During intensive cytological searches in systemically AMV-infected *N. benthamiana* plants no elongated tubules could be discerned, despite an earlier report. At the front of infection modified plasmodesmata were found containing both the AMV CP and MP, and having a significantly wider diameter than those in non-infected as well as fully infected tissues (Chapter 4). This finding implied that the modification of the plasmodesmata by AMV is only transient, restricted to the front of infection. Cryo-sectioning of such plasmodesmata revealed the presence of rows of virus particles within the interior of the plasmodesmal pore, which might suggest that only short, not-extending tubules are formed (Chapter 4 and Figure 1 of General Discussion)

Furthermore, not only a transient modification of plasmodesmata was observed in AMV-infected leaf tissue, also a temporary increase of their number at the infection front was noted (Chapter 5). By inspecting cell walls in mesophyll tissue respectively in front of, at, and after the infection zone it was calculated that the density of plasmodesmata in the infection zone at least doubled. It remains to be resolved whether this increase is due to specific viral induction of new plasmodesmata to become engaged in viral transport or whether this reflects a host response to restore cell-to-cell communication after a major part of the pre-existing plasmodesmata have become modified.

In a simultaneously executed PhD project within the same programme (by L. Jongejan, PhD thesis, University of Leiden, in preparation), the AMV MP was used as bait in the yeast two-hybrid system to identify host (*N. benthamiana*) proteins which are specifically targeted by this viral protein during the infection process. Chapter 6 deals with the cytological analysis of the AD3 protein, an AMV MP-binding plant protein of which the expression could be verified in plant tissue. Specific antibodies were raised against heterologously expressed AD3 and used in immuno-cytological localisation studies. These studies revealed that AD3 is specifically found in membrane fractions of both leaf and root tissues of *N. benthamiana*, and immuno-gold EM demonstrated its localisation in the plasma membrane, which is not in contradiction with a potential function of this protein to support intercellular movement of viruses. Probably due to the low expression levels of AD3 and the transient presence of AMV MP solely at the infection front, a co-localisation between these two proteins *in situ* could not be established.

Samenvatting

Om een volledige infectie van een plant tot stand te brengen, dienen plantenvirussen in staat te zijn om zich na vermenigvuldiging in de primair geïnfecteerde cel te verspreiden naar de omringende, nog niet geïnfecteerde cellen. Het proces van systemische verspreiding van een virus door een plant kan ruwweg ingedeeld worden in twee deelprocessen: cel-tot-cel transport, waarbij plasmodesmata een sleutelrol spelen, en "lange-afstand transport" waarbij het vaatsysteem, met name het floeem een cruciale rol speelt. Het in dit proefschrift beschreven onderzoek richtte zich op het eerstgenoemde transportproces.

Voor cel-tot-cel transport moet het virus de rigide celwand passeren en het ligt voor de hand dat het virus daarbij gebruikmaakt van de z.g. plasmodesmata, de poriën door de celwand waarmee buurcellen t.b.v. intercellulaire communicatie symplastisch met elkaar verbonden zijn. Tot dusver zijn er twee mechanismen beschreven waarmee plantenvirussen in staat zijn hun deeltjes dan wel hun genoom via specifiek gemodificeerde plasmodesmata naar buurcellen te transporteren. Modificatie, in de vorm van vergroting van de doorlaatbaarheid (*size exclusion limit;* SEL), is nodig omdat plasmodesmata normaliter niet permeabel zijn voor macromoleculen groter dan 1 kilodalton (2.5 nm doorsnede). Het mechanisme waarmee tabaksmozaiekvirus (TMV) plasmodesmata modificeert behelst een minimale morfologische verandering van de plasmodesma gepaard gaand met een duidelijke toename van de SEL, zodat het niet-ingepakte virale genoom doorgelaten kan worden. Andere virussen, zoals b.v. het koebonenmozaiekvirus (*cowpea mosaic virus*; CPMV), transporteren hun complete virusdeeltje en dienen derhalve het plasmodesma ingrijpender te modificeren: er wordt een transportbuis aangelegd die via het plasmodesma in de nabuurcel penetreert.

Beide mechanismen worden bewerkstelligd door een viraal gecodeerd eiwit, het z.g. transporteiwit.

Een van de doelstellingen van het hier beschreven onderzoek, dat deel uitmaakte van een door de stichting ALW gesubsidieerd onderzoeksprogramma, was om virale transporteiwitten toe te passen als sonde om via hun interactie met plasmodesmata meer te weten te komen over de eigenschappen en structuur van deze enerzijds zo belangrijke maar anderzijds zo onbekende communicatiekanalen. Tevens zou dit onderzoek aldus meer licht werpen op door virussen gebruikte transportmechanismen binnen de plant. Voor dit onderzoek werden twee verschillende virussen geselecteerd, te weten het luzernemozaiekvirus (*alfalfa mosaic virus;* AMV) en het eerder genoemde CPMV, waarvan verondersteld werd dat deze binnen een gemeenschappelijke gastheer (*Nicotiana benthamiana*) gebruik zouden maken van de twee genoemde transportmechanismen en waarvan derhalve gedacht werd dat hun transporteiwitten op verschillende wijzen een interactie met plasmodesmata zouden ondergaan. Van CPMV was immers al eerder door ons vastgesteld dat dit als complete virusdeeltjes via tubulaire structuren door plasmodesmata getransporteerd werd, terwijl van AMV, op grond van genetische verwantschap met TMV, verondersteld werd dat dit in de vorm van niet ingepakt viraal genoom getransporteerd werd.

In de eerste experimentele hoofdstukken van dit proefschrift (Hoofdstukken 2 -5) wordt allereerst het transportmechanisme van AMV nader gekarakteriseerd, met name de wijze waarop het AMV transporteiwit in staat is plasmodesmata te modificeren. Gebruikmakend van een protoplastensysteem werd allereerst vastgesteld (Hoofdstuk 2) dat, verrassend genoeg, het AMV transporteiwit in staat is tubulaire structuren

gevuld met intacte virusdeeltjes te vormen aan de oppervlakte van de plantencel, geheel analoog aan wat eerder met CPMV gevonden was. Om verdere ondersteuning te verkrijgen voor de veronderstelling dat deze buisvormige structuren betrokken zijn bij cel-cel transport van AMV werd een aantal specifieke virusmutanten gestoord in hun systemische verspreiding nader onderzocht (Hoofdstuk 3). Twee van zulke mutanten (SP6 en SP7), gemuteerd in hun transporteiwit, waren niet meer in staat om dergelijke tubulaire structuren in protoplasten te vormen, en een manteleiwit-mutant (CP4P) die gestoord was in de aanmaak van (stabiele) virusdeeltjes, kon slechts lege buizen produceren. Deze resultaten ondersteunen de conclusie dat succesvol cel-tot-cel transport samenhangt met het vermogen van AMV om tubulaire structuren gevuld met virusdeeltjes te maken, hetgeen dus betekent dat AMV van eenzelfde transportmechanisme gebruik maakt als CPMV. Als preliminair nevenresultaat werd vastgesteld dat alle drie mutanten weliswaar gestoord waren in hun cel-tot-cel transport in parenchymweefsel, maar tot op zekere hoogte nog in staat waren tot gelimiteerd intercellulair transport binnen cortexweefsel. Dit verschijnsel werd in het kader van dit promotieonderzoek niet verder onderzocht maar suggereert dat AMV binnen verschillende weefsels wellicht gebruik kan maken van alternatieve transportmechanismen, iets dat ook voor het verwante komkommer mozaiekvirus (CMV) verondersteld wordt.

Indien het belangrijkste transportmechanisme van AMV het overbrengen van complete virusdeeltjes via tubulaire structuren behelst, mag verwacht worden dat deze buizen, aanwezig zijn in plasmodesmata van geïnfecteerd plantenweefsel. Uitgebreide cytologische analyses (Hoofdstuk 4) toonden aan dat in geïnfecteerde *N. benthamiana* bladeren uitsluitend aan het (voortschrijdende) front van infectie de structuur van plasmodesmata aanzienlijk gemodificeerd wordt: de desmotubule wordt verwijderd en de diameter vergroot. In enkele gevallen werd een rij van virusdeeltjes binnen de plasmodesmale porie aangetroffen, een rangschikking die wees op de aanwezigheid van een buisvormige structuur. Op basis van de verkregen resultaten werd verondersteld dat AMV binnen plantenweefsel inderdaad van cel-tot-cel verspreidt als complete virusdeeltjes via korte (niet langer dan de plasmodesma zelf) buisvormige structuren die bovendien slechts plaatselijk en tijdelijk, aan het front van infectie, worden geproduceerd.

Verder bleek dat aan dit front van infectie het aantal plasmodesmata met een factor 2 a 3 toeneemt, waarvan slechts een deel (ruwweg de helft) gemodificeerd bleek te zijn (Hoofdstuk 5). Het kon vooralsnog niet vastgesteld worden of deze toename actief geïnduceerd was door het AMV (transporteiwit) of dat hier sprake was van een nieuwe aanmaak door de plant als gevolg van de modificatie van (en dus voor normaal cel-cel contact wellicht onbruikbaar geraakte) bestaande plasmodesmata.

In het laatste experimentele hoofdstuk tenslotte (Hoofdstuk 6) staan cytologische analyses beschreven aan het z.g. AD3 eiwit, een gastheer (*N. benthamiana*) eiwit met affiniteit tot het AMV transporteiwit, zoals vastgesteld d.m.v. het gist "two-hybrid" systeem (zie proefschrift Laurian Jongejan, in voorbereiding). Gebruikmakend van een specifiek antiserum kon de intracellulaire locatie van AD3 vastgesteld worden. Op basis van subcellulaire fractionering werd vastgesteld dat dit eiwit in zowel *N. benthamiana* als in andere plantensoorten voorkomt in membraanfracties, zowel in blad- als in wortelweefsel. D.m.v. immuno-elektronenmicroscopie werd bepaald dat AD3 voorkomt op de plasmamembraan en in z.g. lomasomen, organellen die een rol spelen bij de

celwandvorming. Hoewel een directe interactie tussen AD3 en het AMV transporteiwit in de geïnfecteerde cel, mede door de lage concentratie van de beide eiwitten, niet op grond van co-lokalisatie bevestigd kon worden, is de subcellulaire locatie van AD3 in overeenstemming met de mogelijkheid dat dit gastheereiwit een rol speelt bij het intercellulaire transport van AMV.

Curriculum vitae

Op 09 september 1969 werd ik, Nicole Neeltje van der Wel geboren te Naaldwijk. In 1987 behaalde ik mijn HAVO diploma aan het Stevin College in Den Haag en na het succesvol doorlopen van de Hoger Laboratorium Opleiding, Hogeschool Rotterdam & Omstreken te Delft, begon ik in 1991 de studie biologie aan de Landbouwuniversiteit Wageningen (LUW). Tijdens deze studie heb ik een afstudeervak gedaan aan de Vakgroep Plantencytologie- en morfologie begeleid door Dr Annemie Emons (LUW) en Dr Constant Putman (Universiteit Twente). In dit afstudeervak heb ik de toepassingen van de *Atomic Force Microscope* in de botanie onderzocht. Vervolgens heb ik in de *Plant Cell Biology group* van *Australian National University*, Canberra Australië, onderzoek gedaan naar de rol van microtubili in de positionering van nuclei en de verdeling van cytoplasma in sporangia van *Phytophthora cinnamomi*, begeleid door Dr Sandy Jackson en Dr Adrianne Hardham. Op de vakgroep Fytopathologie van LUW onderzocht ik in een derde afstudeervak de *In situ* lokalisatie van het ipiO-gen product in *Phytophthora infestans* m.b.v. het *gus*-gen reportersysteem, onder begeleiding van Dr Francine Govers en Dr Pieter van West.
In 1995 werd ik aangenomen als OIO bij de vakgroep Virologie onder de supervisie van Prof. Rob Goldbach en Dr Jan van Lent. Het project waaraan ik werkte en waarvan de resultaten in dit proefschrift beschreven staan, was onderdeel van een door SLW gefinancierd programma met de titel *De rol van plasmodesmata in virus transport en cel-cel communicatie*. Inmiddels ben ik vanaf september 1999 werkzaam als post-doc in de groep van Prof. Peter Peters binnen het Nederlands Kanker Instituut te Amsterdam. Hier werk ik aan de ontrafeling van de subcellulaire transportwegen van CD1 antigen presenterende moleculen in relatie tot Mycobacterie infecties.

Nawoord

Toen ik begon op de vakgroep Virologie wist ik relatief weinig van virussen maar dacht dat het eenvoudige levensvormen waren, gegeven het feit dat zij uit slechts enkele eiwitten opgebouwd zijn. Nu weet ik dat die enkele eiwitten op knappe wijze een gastheer kunnen benutten en "aan het werk zetten". Ik vond het fascinerend de werking van dit systeem te bestuderen.

Maar zo'n systeem laat zich niet zomaar kennen en er is heel wat werk verricht door de diverse mensen voordat we enkele van de processen in beeld hadden. Allereerst door promotor Rob die me de Virologie binnengetrokken heeft en steeds een sturende rol had, al was ik soms lastig van een bepaalde richting af te krijgen. Daar had ook Jan last van die, wanneer ik na een sessie achter de elektronen microscoop allerlei conclusies dacht te kunnen trekken waarvan ik beslist overtuigd was (en soms nog ben), mij wist af te remmen met argumenten en realisme. Maar je gaf me vaak ook weer een duwtje in de juiste richting wanneer ik die niet meer zag.

Een eerste goede zet in een richting die essentieel bleek voor de rest van dit proefschrift was de uitspraak: "virussen transporteren via buizen!". Daniella, ik heb veel van je geleerd, en dan bedoel ik niet alleen over transportmechanismen van virussen. Wat ik verder nooit had voorzien was dat de filosofische kanten van virustransport belangrijke inspiratiebronnen konden zijn! Marc, bedankt hiervoor. Inspiring to think, sometimes in between experiments or during a nice diner with a good glass of wine is essential. Päivi and Chris have the talent to inspire at both occasions. Marillia you were always a very pleasant roommate and I wish you all the best during your PhD project. Joop, jij wist altijd precies op het juiste moment weer een oude truc uit de kast te halen om een en ander visueel te maken. Hanke, je hebt enorm hard getrokken aan het transgene plantenwerk en je hebt me geleerd (vind ik tenminste) hoe het lab enigszins gestructureerd te houden! Tim en Christy hebben mij heel goed geholpen door hun projecten heel enthousiast en nauwkeurig uit te voeren. Verder was de samenwerking met Laurian Jongejan, Huub Linthorst en John Bol van de Universiteit Leiden heel erg nuttig en heeft mij veel geleerd over AMV en het Two Hybrid systeem. De mannen van Moleculaire Biologie (Joan, Peter, Jan, Jan en Ab) en Marcel wil ik daarnaast nog even bedanken voor het bijbrengen van enkele beginselen van de moleculaire wetenschap en Peter ook nog voor het paranimf zijn.

Heel wat mensen op en naast de vakgroep zijn belangrijk geweest voor het sociale leven en de gezelligheid waar een OIO niet zonder kan. Dus alle Tospo's, Baculo's, Molbie-mannen, (ex)Viro-vrouwen en andere Afko's: bedankt voor alle eet- en drinkavondjes.

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Tenslotte Wolf, jou bedanken met één zinnetje of pagina zou niet genoeg zijn dus voor jou deze kreet;

HET IS AF!!

Bedankt allemaal,   Nicole.