On the involvement of host proteins in *Cowpea mosaic virus* intercellular spread

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Thesis

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General introduction.

General introduction

Viruses are obligate intracellular parasites, meaning they can only replicate inside the cells of their host. Plant viruses enter their host via wounds inflicted by mechanical damage or by feeding of their vector. To establish a successful infection, viruses need to replicate and spread throughout their host. This spread involves both short distance movement, from the infected cell to neighbouring cells (cell-to-cell movement), and long distance spread to distal parts of the plant. Long distance or systemic spread of viruses occurs via the vascular tissue of the plant. Here, most viruses are transported to developing parts of the plant through the phloem, thus following the route of photo-assimilates (reviewed by Waigmann *et al.*, 2004; Vuorinen *et al.*, 2011). Short distance, or intercellular movement, is required to reach the vasculature from the initially infected cell and is also required for spread of the virus following its release from the phloem in distal parts of the plant. Intercellular transport is hampered by a cell wall that encloses the plant cell (Figure 1). This cell wall provides rigidity to the plant body, protects cells from abiotic and biotic stresses such as water loss and invasion by pathogens, but also regulates the exchange of molecules between cells. Cell walls are bridged by channels, called plasmodesma (PD; plural plasmodesmata, PDs), that enable communication between plant cells. These PDs are also the only conduit for viruses to move between cells and are therefore essential for their intercellular movement. In this chapter current insights in cell-to-cell movement of (plant) viruses will be discussed, with special focus on the mechanism of tubule-guided transport and the role of host components in this process.

Plasmodesmata: intercellular connection channels

PDs are channels in the cell wall that enable communication between cells. In their native state PDs only permit the free diffusion of small molecules (< 1000 Da) and do not allow the movement of larger molecules like proteins, let alone viruses or viral genomes. Therefore, intercellular transport of viruses or viral genomes requires modification of the PD structure (Figure 1). PDs are complex membranous channels which are formed during cell division from strands of the endoplasmic reticulum (ER) that are trapped in the forming cell plate. PDs can also be formed *de novo* at later stages in plant development (Hepler,

1982; Seagull, 1983). The lumen of the PD, the cytoplasmic sleeve, is lined with plasma membrane and contains a strand of tightly compressed ER, called the desmotubule (Figure 2; Roberts, 2007; Bell and Oparka, 2011). The PD channel therefore ensures both membrane and cytosolic continuity between cells. The orifice of the PD is surrounded by a callose collar, which dictates the diameter of the PD neck (Radford *et al.*, 1998). Because PDs are embedded in the ridgid cell wall, the identification of their protein content has been a challenging venture. Immunological studies showed the presence of actin (White *et al.*, 1994), myosin (Radford and White, 1998), centrin (Blackman *et al.*, 1999) and

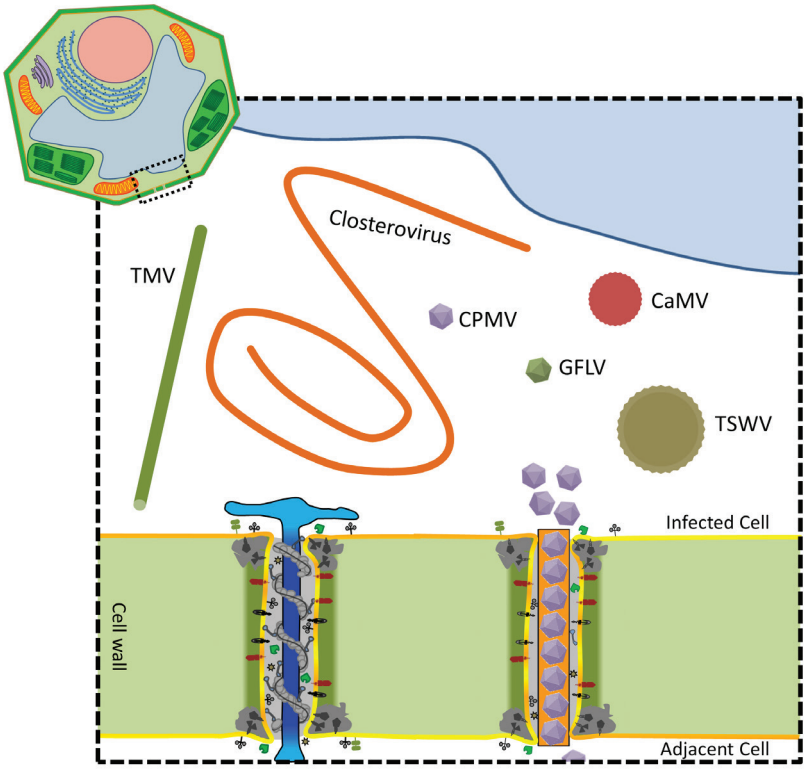

Figure 1: Schematic representation of a plant cell with a detailed view of the cell wall containing two PDs.
Various plant viruses are shown to indicate their size relative to the native PD (left). The PD ensures plasma membrane (yellow) and ER (blue) continuity between adjacent cells. In CPMV infected cells, virion containing movement tubules are assembled from MP inside the PD (right). Insertion of movement tubules in PD requires the removal of the desmotubule (dark blue, in left PD) and other proteinaceous structures from the cytoplasmic sleeve (gray). Abbreviations of viruses depicted: CPMV (*Cowpea mosaic virus*, 28 nm), TMV (*Tobacco mosaic virus*, 300 nm x 18 nm), GFLV (*Grapevine fanleaf virus*, 28 nm), CaMV (*Cauliflower mosaic virus,* 50 nm), TSWV (*Tomato spotted wilt virus*, 80 nm), *Closterovirus* (2500 nm x 10 nm).

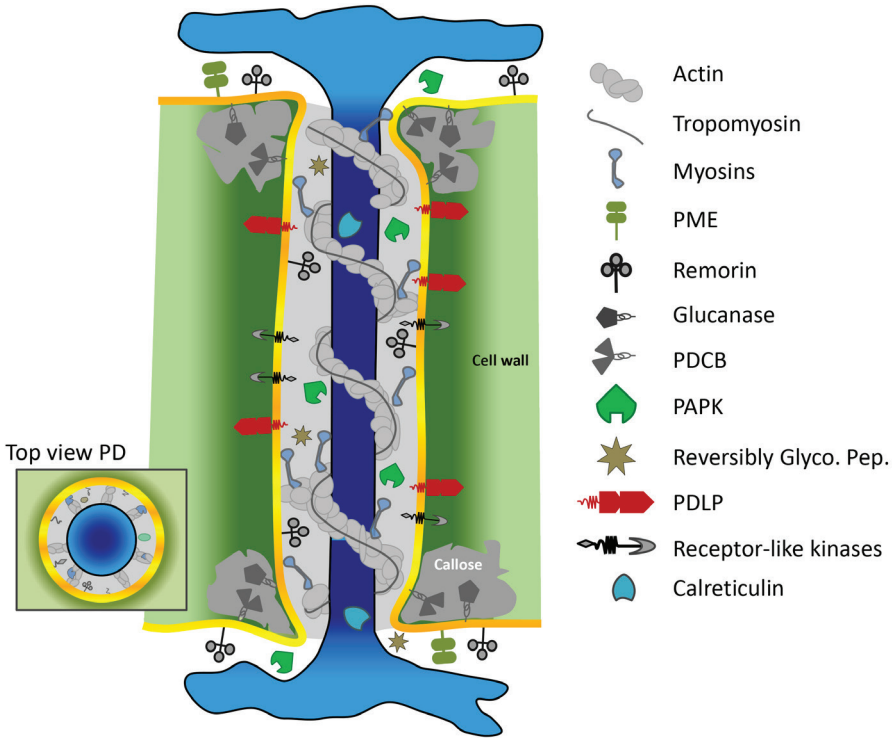

Figure 2: Detailed representation of a plasmodesma.
The predicted location of a selection of known PD-associated proteins (see text) has been indicated. The PD is lined by plasma membrane (yellow) and connects the ER of adjacent cells (blue) by a compressed strand of ER, the desmotubule (dark blue) that transverses the PD pore. A top view of the PD shows how "proteinaceous spokes" segment the lumen of the PD, known as the cytoplasmic sleeve (grey), into micro-channels which restrict the SEL of the PD. The colour gradient in the cell wall surrounding the PD indicates a difference in cell wall composition. Figure modified from Faulkner and Maule (2011).

tropomyosin (Faulkner *et al.*, 2009) in the cytoplasmic sleeve. Actin (possibly in conjunction with myosins) is thought to form spokes that interconnect the plasma membrane and desmotubule, thus creating micro-channels that limit the molecular size of substrates that can pass though the cytoplasmic sleeve to less than 1 nm in diameter (Figure 2; Wolf *et al.*, 1989; Citovsky *et al.*, 1992; Faulkner and Maule, 2011). This permeability of PDs is defined as the size exclusion limit (SEL). Advances in biochemical and proteomic analysis enabled the identification of additional PD-associated proteins in purified cell wall and plasma membrane fractions. PD-callose binding proteins (PDCBs; Simpson *et al.*, 2009), β-1,3-glucanases (Levy *et al.*, 2007), class 1 reversibly glycosylated peptides (Selth *et al.*, 2006), PD-associated protein (casein) kinase I (PAPK; Lee

et al., 2005) and pectin methyl esterase (PME; Dorokhov *et al.*, 1999) are proteins found in cell wall material and are thus associated with the PD. Receptor-like PD-located proteins (PDLP; Thomas *et al.,* 2008), remorin (Raffaele *et al.*, 2009) and receptor-like kinases proteins (Jo *et al.,* 2011) are plasma membrane proteins associated with PDs (Figure 2). The ER-located proteins calreticulin (Baluška *et al.,* 1999) and reticulon (Fernandez-Calvino *et al.,* 2011) have also been detected in the PD. Although the function of the majority of the PD-associated proteins is unknown, altered expression levels of PDCBs, PDLPs, β-1,3-glucanase, reversibly glycosylated peptides and PME are known to alter the molecular fluxes through PDs.

It is obvious that PDs are not just "holes in the wall", as these channels are highly dynamic and their SEL can be modulated upon changes in physiological condition or upon biological stress (reviewed by Maule *et al.*, 2011; Bunkard *et al.*, 2013). Dynamic regulation of the SEL is essential for selective transport of molecules and the formation of intercellular molecular gradients, which are required for growth and development of the plant and partitioning of the cytoplasmic continuum (Guseman *et al.*, 2010; Burch-Smith and Zambryski, 2012; van Norman *et al.*, 2011). The SEL of PDs in developing cells, which import photo-assimilates, is estimated to be up to 50 kDa (Oparka *et al.*, 1999; Waigmann *et al.*, 2004). During differentiation these cells switch from an importing to an exporting state and this switch coincides with a decrease of SEL to less than 10kDa (Oparka *et al.*, 1999), hence severely limiting the exchange of substrates between cells.

The SEL dictates the size of molecules that can be transported via PDs and therefore needs to be adjusted to enable transport of large (>10 kDa) endogenous or viral proteins and complexes. The molecular mechanisms that regulate SEL dynamics are not fully understood. However, enhanced callose deposition was found to decrease PD SEL (Northcote *et al.*, 1989), as was silencing of the callose degrading β-1,3-glucanase enzymes (Iglesias and Meins, 2000), indicating a relation between the SEL and callose deposition at the PD neck region (Figure 2). Since viruses are spread between plant cells and native PDs do not even permit the passage of naked viral RNA (with an estimated Stokes radius of 10 nm for TMV RNA; Citovsky *et al.*, 1992), viruses are apparently successful in modifying PDs to enable their passage. To this end, plant viruses encode specialized proteins called movement proteins (MP).

Movement of viruses between cells

Viral MPs are highly versatile and their functions in intercellular transport include: binding of nucleic acids, localization and accumulation at the PD, increasing PD SEL, interactions with the cytoskeleton, the ability to traverse the PD and assembly of movement tubules (Meshi *et al.*, 1987; Wolf *et al.*, 1989; Citovsky *et al.,* 1990; Waigmann and Zambryski, 1995; Melcher, 2000). The mode of action of MPs differs per virus family and the functions necessary for successful intercellular movement may be combined into one MP or may be divided over several MPs. Based on the number of proteins and their functional domains, MPs are classified into different superfamilies (Melcher, 2000). Some of the best characterized MPs belong to the so-called 30K superfamily. Members of this superfamily share conserved secondary structures and functional domains, but do not necessarily share high degrees of amino acid similarity (Mushegian and Koonin, 1993; Melcher, 2000). These MPs have their movement functions combined in a single protein and transport viral genomes between cells either in an encapsidated or non-encapsidated form.

Transport of encapsidated genomes, i.e. virions, has long been known. Arrays of particles have been observed in the PD of cells infected with viruses of the families *Secoviridae* (van der Scheer and Groenewegen, 1971; Kim and Fulton, 1971; Šarić and Wrischer 1975; Murant *et al.*, 1975; Wieczorek and Sanfaçon, 1993; Stussi-Garaud *et al.*, 1994), *Bromoviridae* (Francki *et al.*, 1985; Martelli and Russo, 1985; van der Wel *et al.,* 1998; Grieco *et al.*, 1999), *Geminiviridae* (Kim and Lee, 1992) and *Caulimoviridae* (Kitajima *et al.*, 1969; Linstead *et al.*, 1988; Cheng *et al.,* 1998). Virions were found to be transported through PDs via tubular structures composed of the viral MPs (Wellink *et al.*, 1993; Wieczorek and Sanfaçon 1993; Perbal *et al.*, 1993; Kasteel *et al.*, 1997). This mode of transport is known as tubule-guided intercellular transport and is exemplified by the *Comovirus Cowpea mosaic virus* (CPMV; Figure 3; van Lent *et al.*, 1990) and the *Nepovirus Grapevine fanleaf virus* (GFLV; Ritzenthaler *et al.*, 1995). Viruses that move between cells in a non-encapsidated form often rely on the formation of a ribo-nucleoprotein complex (vRNP) consisting of the RNA genome and various viral proteins including the MP (Citovsky *et al.*, 1992; Kawakami *et al.*, 2004). This type of intercellular transport is exemplified by *Tobacco mosaic virus* (TMV).

The role of movement proteins in intercellular transport of viruses

The 30 kDa protein of TMV was the first plant viral protein that was identified to be a MP and is the type member of the 30K superfamily. The requirement of this protein for cell-to-cell movement was established by generating mutations in the MP, which resulted in reduced spread of the virus without hampering its replication (Meshi *et al.*, 1987). Complementation of the movement deficient virus with a wild type 30 kDa protein restored the ability of the mutant virus to move between cells, showing that the 30 kDa protein was indeed essential for virus movement (Deom *et al.*, 1987; Meshi *et al.*, 1987). Transient expression of TMV MP showed that the protein localised to the PD (Atkins *et al.*, 1991; Ding *et al.*, 1992) and that it increased the SEL of the PD (Wolf *et al.*, 1989; Waigmann *et al.,* 1994). The discovery that TMV MP tightly binds viral RNA in a sequence-unspecific manner (Citovsky *et al.*, 1990) and that the protein is able to move between cells (Waigmann and Zambryski, 1995) led to the hypothesis that MPs facilitate intercellular transport by both modification of the PD architecture and chaperoning viral genomes through the modified PD. The molecular mechanisms by which TMV MP orchestrates the passage of the vRNP complex through dilated PD are not fully understood. However, TMV MP has actin-severing abilities which could increase PD SEL by removal of actin spokes from the cytoplasmic sleeve (Su *et al.*, 2010). Furthermore, this MP also induced callose degradation at the PD by recruiting β-1,3-glucanases (Guenoune-Gelbart *et al.,* 2008; Epel, 2009; Ueki *et al.*, 2010).

With viruses that move in form of mature virus particles, the MP assembles into a tubular structure within the PD pore. Such tubules carry virions to adjacent cells. In contrast to the transport of vRNP complexes, tubule-guided intercellular movement requires more extensive structural rearrangement of the PD. The desmotubule and other structural features must be removed from the PD pore to enable insertion of the virally encoded movement tubule (Figure 1; Davison, 1969; de Zoeten and Gaard, 1969; van der Scheer and Groenewegen, 1971). Insertion of the movement tubules of *Alfalfa mosaic virus* and *Cauliflower mosaic virus* (CaMV) requires dilation of the PD channel (Linstead *et al.*, 1988; van der Wel *et al.*, 1998) and therefore these viruses also need to modify the cell wall surrounding the PD. In analogy to the dynamics of microtubule growth and shrinkage, the assembly and disassembly of movement tubules is possibly

assisted by interactions of the MP with GTP. With microtubules, incorporation of GTP stabilizes the growing tubules (Quiniou *et al.*, 2013). Similarly, GTP-binding of the CPMV MP could stabilize the formation of the movement tubule (Carvalho *et al.*, 2004).

Some fundamental characteristics of tubule formation have been determined in protoplast studies. Protoplasts are isolated plant cells devoid of their cell wall. Nonetheless infection of protoplasts with tubule-forming viruses leads to extensive formation of movement tubules, even though PDs are absent (van

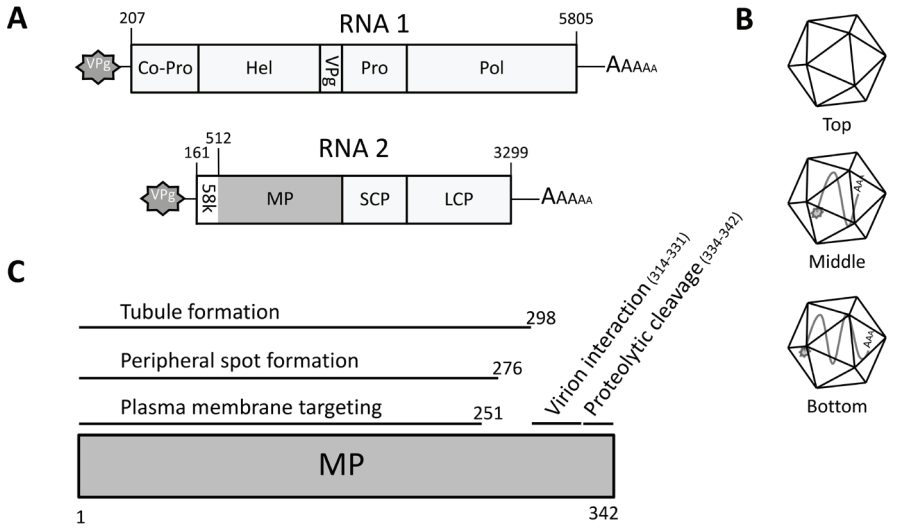

Figure 3: The *Cowpea mosaic virus*, its RNA genome, its particles, and the functional domains of its movement protein.

A) Genetic make-up of the bipartite genome. Both RNA molecules have a VPg (viral protein genome-linked) at their 5'-end and a poly-A tail at their 3' terminus for efficient transcription. RNA1 is translated in one large polyprotein that is proteolytically cleaved by the protease (Pro) and a co-factor (Co-Pro), these proteins govern the release of functional polymerase (Pol), helicase (Hel) and VPg (Goldbach and Wellink, 1996). RNA2 encodes two polyproteins using different start codons (at nucleotide 161 and 512). Both polyproteins contain the small and large coat proteins (SCP and LCP respectively) and either a 58 kDa protein or the 48 kDa MP. **B)** Representation of the three CPMV particles formed during infection, Top, Middle and Bottom particles containing no RNA, RNA 2 and RNA 1 respectively. **C)** Functional domains of the MP. The regions that are essential for tubule formation, peripheral punctate spot formation and plasma membrane targeting are indicated. Amino acids 314 to 331 are essential for specific interactions between the tubule and virions and amino acids 334 to 342 are required for proteolytic cleavage. (Figure adjusted from Pouwels, 2004)

Lent *et al.*, 1991; Ritzenthaler *et al.,* 1995; Kasteel *et al.,* 1997). These tubules protrude into the culture medium and are engulfed by the plasma membrane (Figure 4; van Lent *et al.*, 1991; Perbal *et al.,* 1993; Storms *et al.*, 1995; Kasteel *et al.*, 1997). Transient expression of MPs is sufficient to induce tubule formation, demonstrating that the MP is the only viral protein required for tubule formation (Wellink *et al.*, 1993; Storms *et al.*, 1995; Zheng *et al.*, 1997).

Tubule-guided intercellular transport of CPMV

Tubule-guided intercellular transport has been extensively studied with CPMV, the type species of the genus *Comovirus* from the family *Secoviridae.* Viruses from this family form small, 28-30 nm icosahedral particles, which contain a bipartite positive-sense RNA genome (Figure 3A). The two RNA molecules are encapsidated separately (Figure 3B) and are both required for infection of the host. RNA1 encodes the proteins required for replication and RNA2 codes for the 58K protein and the three proteins required for viral spread: the 48K MP, the large and the small coat protein (Figure 3A; Wellink and van Kammen, 1989). Replication of CPMV occurs in the cytoplasm at membranous vesicles derived from the ER, which are surrounded by electron dense proteinacious material (Figure 4C; Carette *et al.*, 2000). Progeny virus particles accumulate in the cytoplasm and at the periphery of the cell these virions are incorporated in movement tubules assembled from MP (Figure 4).

The characteristics of the MP have been studied over the last decades and a number of the functional domains have been identified (Figure 3A). A large segment of the MP (amino acid (aa) 1 to 298) appears to be essential for tubule formation, the region from aa 1 to 276 is required for accumulation of MPs into peripheral punctate spots at the plasma membrane and aa 1 to 251 are required for plasma membrane targeting of the MP (Figure 3C; Lekkerkerker *et al.*, 1996; Pouwels *et al.*, 2003). The C-terminal part of the MP (aa 314 to 331) is essential for virion binding (Lekkerkerker *et al.*, 1996) and is located on the inside of the movement tubule (Bertens *et al.*, 2003). C-terminal addition of GFP resulted in the formation of movement tubules that did not contain virions, likely because the GFP fusion prevented the interaction between the MP and the virion (Bertens *et al.*, 2003). The interaction of the MP with the CPMV virion appears to be highly specific, since the MP only interacted with the large coat protein and did not bind

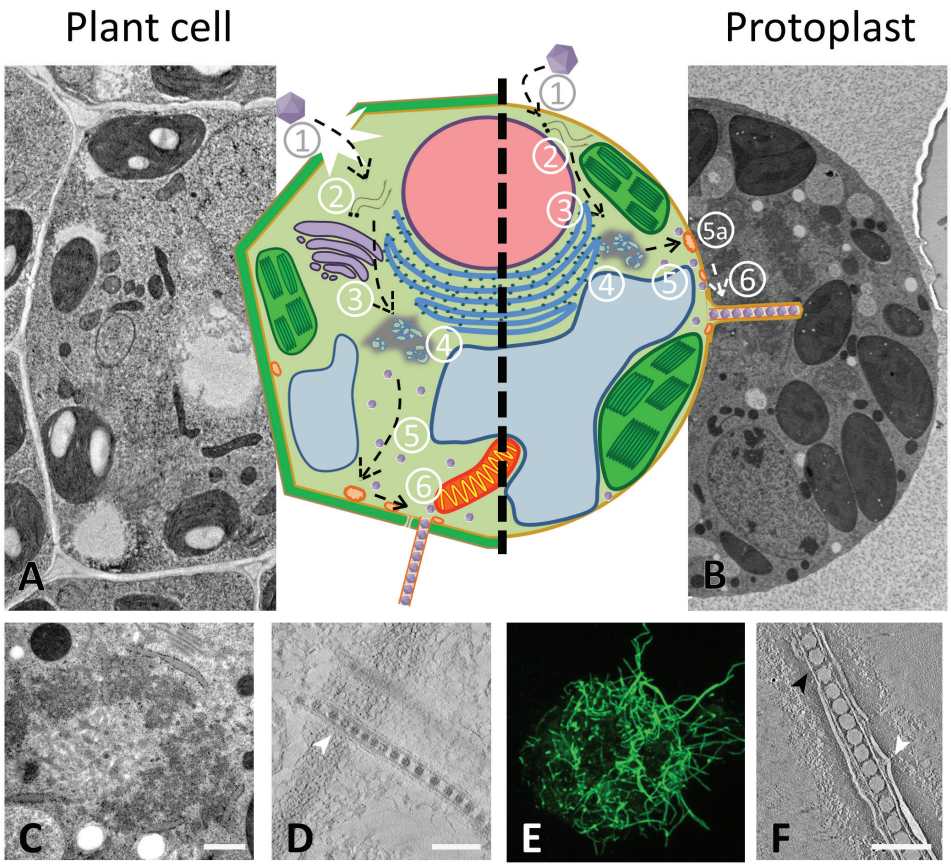

Figure 4: CPMV infection and tubule formation in plant cells and protoplasts.
Schematic representation of the progression of CPMV infection: Virus particles enter the cell (1) where they disassemble and release the RNA genome in the cytoplasm (2). Host factors readily translate the CPMV genome into the viral poly-proteins, which after proteolytic processing, initiate to the formation of cytopathic structures (3). In these ER-derived vesicular structures replication of the virus takes place (4). Progeny virus assembles and accumulates in the cytoplasm (5). In protoplasts MPs accumulate at the plasma membrane and form peripheral punctate spots (5a). At the PD (and from peripheral punctate spots in protoplasts) the movement protein assembles into tubular structures (6) that transport the CPMV particles to adjacent cells (in the plant), or which grow out into the culture medium engulfed by the plasma membrane (in protoplasts). **A)** Electron microscopy (EM) image of a CPMV infected mesophyll cell of cowpea (*Vigna unquiculata*) **B)** EM image of a cowpea protoplast infected with CPMV **C)** Detailed EM image of cytopathic structures in which CPMV replicates **D)** Electron tomographic reconstruction of a CPMV movement tubule inserted in the PD (white arrow head) **E)** Immuno-fluorescent labelling of tubules protruding from an infected protoplast. **F)** Cryo-EM image of isolated movement tubule (black arrowhead) that is engulfed by plasma membrane (white arrowhead). Scale bar in C is 500 nm and in D and F 100 nm. Protoplast diameter (in E) is 20 µm.

coat proteins of other plant viruses (Carvalho *et al.*, 2003).

It is unknown by which route the virions or the MPs reach the PD. Considering the abundance of virions in the cytoplasm of infected cells, it is conceivable that virus particles passively translocate to the PD. For the translocation of MPs diffusion certainly is an option as molecules of up to 500 kDa diffuse efficiently through the cytoplasm (Seksek *et al.*, 1997) and a functional endomembrane system or cytoskeleton was shown to be dispensable for CPMV MP targeting to the plasma membrane (Pouwels *et al.,* 2002). Studies in protoplasts using C-terminal MP-GFP fusions have shown that tubule formation is a stepwise process. The process starts with accumulation of the MP at the plasma membrane in so-called peripheral punctate spots. The catalyst for this localised accumulation is unknown but could well be a PM-associated protein. From these peripheral punctate spots tubule formation is initiated (Figure 4; Pouwels *et al.*, 2004).

The mechanism by which the MP, inevitably in concert with host factors, modifies the PD to allow tubule insertion and subsequent translocation of virions is still largely unknown and subject of this thesis research. Processes such as tubule anchoring, tubule assembly, incorporation and release of virions from the tubule, are poorly understood and undoubtedly rely on both viral and host factors. Currently, no host proteins have been identified that interact with the CPMV MP during infection or intercellular transport. The formation of tubules upon expression of MP in insect cells (Kasteel *et al.*, 1996), thus in cells that do not support CPMV infection nor have even remnants of PD, indicates that the host proteins required for tubule formation at the plasma membrane must be conserved between the plant and animal kingdoms.

Host proteins and viral transport

Understanding the role of host proteins in intercellular transport requires knowledge of the intimate interaction between such proteins and viral MPs, as these interactions have been shown to orchestrate the movement of viruses between cells. MPs interact with a broad range of host proteins from various cellular pathways and compartments (Table 1). Interactions of MPs with components of the cytoskeleton and the endomembrane system are known and likely relate to the targeting of MPs to the PD. Interactions found between plasma membrane or PD-proteins and MPs could serve the recognition of the PD

or the assembly of movement tubules inside the PD. In the following section an overview of the interactions between MPs and host proteins and the relevance (if known) of these interactions for tubule-guided transport will be presented.

For intercellular movement of CPMV the cytoskeleton does not seem to be required, as disruption of either the microtubule or the actin cytoskeleton in protoplasts does not hamper tubule formation (Pouwels *et al.*, 2002). However, this independence from the cytoskeletal system is not a common feature of all tubule-forming viruses. In tobacco BY-2 cells that expressed the 2B MP of GFLV, depolymerisation of microtubules caused aberrant localization of movement tubules, suggesting that this virus requires intact microtubules for tubule formation at the PD. In the same cells MP function was not affected by actin depolymerisation (Laporte *et al.*, 2003). In contrast, tubule formation by CaMV was unperturbed by disruption of microtubule organisation (Huang *et al.,* 2000), but was hampered when the actin cytoskeleton was disrupted (Harries *et al.*, 2009a). Although microtubules and microtubule binding proteins such as EB1 (Brandner *et al.*, 2008) and MPB2C (Kragler *et al.*, 2003) have been demonstrated to influence the transport of TMV (Kawakami *et al.*, 2004; Ouko *et al.*, 2010) and an intact actin cytoskeleton has been shown to be required for the intercellular transport of other non-tubule-forming viruses (*Potato leafroll virus*, Vogel *et al.*, 2007; *Barley stripe mosaic virus*, Lim *et al.*, 2013; *Turnip vein clearing virus*, Levy *et al.,* 2013), knowledge on the role of the cytoskeleton in tubule-guided movement is limited and may be specific to individual virus-host combinations.

The involvement of the cytoskeleton in intercellular transport does not necessarily require a direct interaction between MPs and cytoskeletal proteins, such as myosin and kinesin motor-proteins. These proteins direct vesicle transport along the cytoskeleton, have also been found to interact with viral proteins. Kinesin-like proteins have been shown to associate with the MP of the tubule-forming virus *Tomato spotted wilt virus* (TSWV, von Bargen *et al.*, 2001). Myosins are also required for PD localization of *Tenuivirus* MP (Yuan *et al.*, 2011), *Closterovirus* particles (Avisar *et al.*, 2008) and are directly or indirectly required for intercellular transport of various other viruses (TMV, Harries *et al.*, 2009b; TSWV, Feng *et al.*, 2013; *Turnip mosaic virus*, Agbeci *et al.*, 2013). This suggests that the dependence of virus intercellular transport on the actin microfilament network is often governed by interactions of MPs with myosins. For the tubule-

forming virus GFLV, Amari and co-workers (2011) have elegantly shown that the involvement of myosin XI, but not myosin VIII, was required for efficient intercellular transport. These authors demonstrated that myosin XI facilitated the targeting of the PD-located proteins (PDLPs) to the PD. As it was concluded that correct PDLP localisation was required for docking of the MP at the PD, myosin proteins thus indirectly regulate the intercellular movement of GFLV (Amari *et al.*, 2011). This example illustrates the complex interactions between actin, myosin and the transport of vesicles and certain viruses.

Secretory vesicles and their cargo, transported along the cytoskeleton, are possible vehicles for direct transport of MPs to the plasma membrane and the PD. Associations between intracellular vesicular trafficking and tubule-guided virus transport are found in the interactions between the MP of CaMV and the *Arabidopsis thaliana* rab-acceptor-like protein MPI7 (Huang *et al.*, 2001) and the interaction of GFLV 2B with the syntaxin KNOLLE (Laporte *et al.*, 2003), which function in endocytosis and vesicle fusion, respectively. Involvement of vesicle trafficking in intercellular transport is further supported by the interaction of the MPs of TMV and several *Begomoviruses* with synaptotagmins, well-known regulators of endo- and exocytosis (Lewis and Lazarowitz, 2010) and the interaction of the TGB2 movement protein of *Potato mop-top virus* with an exocytosis related DNA-J protein (Haupt *et al.*, 2005).

Besides proteins from the cytoskeleton and endomembrane systems, endogenous chaperones from the heat shock protein (HSP) family also interact with MPs and regulate intercellular transport. The *Closterovirus Beet yellows virus* does not form tubules, but encodes an HSP70 homologue which is essential for localization of the virion to the PD and for transport of this virus to adjacent cells (Alzhanova *et al.*, 2001). The tubule-forming MP of TSWV associates with an DNA-J like protein (Soellick *et al.*, 2000), a known co-chaperone of HSP70s. The HSP70 co-chaperones HSP40 and DNA-J proteins were also found to interact with the MPs from TMV (Shimizu *et al.,* 2009; Du *et al.*, 2013) and *Rice stripe virus* (Lu *et al.*, 2009). The spread of *Abutilon mosaic virus* is enhanced by an interaction between its MP and HSP70 chaperones (Krenz *et al.*, 2010). This suggest that a complex of HSP70 and ancillary co-chaperones could assist in either the localisation of MPs towards the PD or that these chaperones could facilitate the formation of protein complexes allowing entrance of viral transport structures

to the PD (Alzhanova *et al.*, 2001; Dolja *et al.*, 2006; Niehl and Heinlein, 2011). However, experimental data clarifying the precise mode of action of HSPs is lacking.

The PD is a bottleneck in virus intercellular transport. As all viruses need to modify the PD structure to enable their transport to neighbouring cells, it is evident that cell wall proteins, plasma membrane located proteins and PD-specific proteins are potential interactive partners of MPs. Interaction between the cell wall-localised protein pectin methyl esterase (PME) and the MPs of *Turnip vein clearing virus*, TMV and CaMV have been demonstrated *in vitro* (Dorokhov *et al.*, 1999; Chen *et al.*, 2000), where PME is thought to facilitate targeting of the TMV vRNP-complex to the PD (Dorokhov *et al.*, 1999; Chen *et al.*, 2000; Waigmann *et al.*, 2004; Lionetti *et al.*, 2014). However, *in vivo* interactions with PME have only been shown for the MPs of TMV and *Chinese wheat mosaic virus* (Andika et al., 2013), viruses that do not form tubules. Additionally, the MPs from TMV and *Begomoviruses* interact with PD-associated kinase protein (TMV and *Bean dwarf mosaic virus*, Lee *et al.*, 2005) and PD-localised reversibly glycosylated peptides (TMV and *Tomato yellow leaf curl virus*, Selth *et al.*, 2006), host proteins with a negative effect on the intercellular spread of TMV (Lee *et al.*, 2005; Zavaliev *et al.*, 2010). Two other host proteins that interact with TMV MP at the PD are the plasma membrane protein remorin (Raffaele *et al.*, 2009) and the ER-localised protein calreticulin (Chen *et al.*, 2005). Overexpression of calreticulin, like the microtubule-associated protein MPB2C, resulted in translocation of MPs away from the PD towards the microtubules. This MP translocation may be a defence response of the cell required for the proteolysis and eradication of MPs (Niehl and Heinlein, 2011), however, this has not been verified experimentally.

To date the only PD-located interactions found between host proteins and tubule-forming MPs are those with the PD-located proteins (PDLPs, *Amari et al.*, 2010) and the recently discovered interaction with patellins 3 and 6 (Peiro *et al.*, 2014). However, only the former interaction with PDLPs is known to aid tubule-guided intercellular spread of viruses. These PDLPs interact with the MPs of GFLV and CaMV in the PD and down-regulation of PDLPs reduced the tubule forming ability of these viruses (Amari *et al.*, 2010). The observation that inhibition of PDLP targeting to the PD resulted in diffusion of the GFLV MP throughout the cytoplasm suggests that PDLPs do not only benefit tubule formation but could

also be involved in localisation of MP to the PD (Amari *et al.*, 2011).

Outline of this thesis

Over the last decades an array of MP-interacting host proteins has been discovered (Table 1) and a broad spectrum of functions has been assigned to these interacting proteins. But how these proteins contribute to successful local and systemic viral infection remains elusive. Also, the mechanistic aspects of tubule-guided transport of viruses, an important step in the spread of virus infection, remain to be clarified. For example, how does the MP translocate to the PD? Are peripheral punctate spots formed *in planta*? How is the movement tubule anchored in the PD? Which host proteins associate with the movement tubule, and how do these modify the existing PD channels? To address these issues and to gain insight in the host components associated with CPMV infection, this thesis research focuses on the role of host proteins in the intercellular movement of CPMV.

Chapter 2 describes a study on the possible role of plasma membrane intrinsic proteins (PIP) in CPMV intercellular movement. These proteins have previously been suggested to interact with the MP and their possible involvement in intercellular transport was tested by co-localisation studies with the CPMV MP and by assessment of tubule-formation in silenced protoplasts.

In **Chapter 3** a proteomic inventory is made of proteins that are present in the plasma membrane-movement tubule complex. These complexes were isolated from infected protoplasts and after purification analysed by tandem-mass spectrometry. Host proteins identified in this complex are candidates for a function in tubule-guided movement, and their role as such is discussed. A selection of host proteins identified in chapter 3 is further characterised in **Chapter 4**. Their involvement in CPMV infection and intercellular movement is investigated by targeted gene silencing assays.

PDLP is the only host protein known to date that aids in the formation of movement tubules. In **Chapter 5** the role of PDLP in CPMV intercellular transport is investigated. Localization of fluorescently labelled PDLP and MP and the formation of tubules is studied in plant tissues (with PDs) as well as in isolated

protoplasts (without PDs). Interactions between PDLP and MP are analysed by fluorescent life-time (FLIM) imaging.

In **Chapter 6** the implications of the work presented in this thesis are discussed in light of the current literature on tubule-mediated viral transport. A model for the tubule-guided movement of CPMV is presented.

Table 1: Host proteins known to interact with movement proteins.

Host protein	Interaction with MP of:	Category	Predicted function in virus transport	Reference
PDLP 1-8	GFLV, CaMV, CPMV	Plasmodesmal protein	Tubule initiation in PD	Amari *et al.*, 2010 This thesis (Chapter 5)
NtPME	TMV, TYLCV, CaMV	Plasmodesmal protein	Translocation of MP to the PD.	Chen *et al.*, 2000 Dorokhov *et al.*, 1999
Calreticulin	TMV	Plasmodesmal protein	MP localisation to the microtubules	Chen et al., 2005
PAPK	TMV, BDMV	Plasmodesmal protein	Phosphorylation of MP	Lee *et al.*, 2005
Cell wall-associated phosphatase	TMV, PLRV	Cell wall protein	Regulation of MP translocation	Citovsky et al. 1993; Karpova et al. 1997; Waigmann et al. 2000;
SlUPTG1	TYLCV	Cell wall / Plasma membrane protein	Glycosylation of MP	Selth *et al.*, 2006
ATP8	TCV	Plasma membrane protein	Unknown	Lin and Heaton, 2001
Tubilin	TMV, BYV	Cytoskeleton component	Regulation of translocation and degradation of MP	McLean *et al.*, 1995 Heinlein *et al.*, 1995,
Gamma- tubulin	TMV	Cytoskeleton component	Translocation of MP-complexes along microtubules	Sambade et al., 2008
Actin	TMV, CMV	Cytoskeleton component	Transport of MP to the PD	McLean *et al.*, 1995 Su *et al.*, 2010
EB1a	TMV	Cytoskeleton component	Translocation of the MP or MP-containing complexes	Brandner *et al.*, 2008
MPB2C	TMV	Cytoskeleton component	Localisation of MPs to microtubules, for protein degradation	Kragler *et al.*, 2003
At4-1	TSWV	Cytoskeleton component	HSP70 mediated cytoskeleton interactions	Von Bargen *et al.*, 2001
NtCDC48	TMV	Endomembrane system	Degradation of MP to regulate accumulation and spread of the virus	Niehl *et al.*, 2012
AtCDC48b	TMV, ORMV	Endomembrane system	Degradation of MP to regulate accumulation and spread of the virus	Niehl *et al.*, 2012

Table 1: Host proteins known to interact with movement proteins. (continued)

Host Protein	Interacts with MP of:	Category	Predicted function in virus transport	Reference
Synaptotagmin	CaLCuV, SLCV, TMV	Endomembrane system, Vesicle transport	Transport of MPs to the PD	Lewis and Lazarowitz, 2010
KNOLLE	GFLV	Endomembrane system, Vesicle transport	Targeting of MPs to the (dividing) cell plate	Laporte et al., 2003
MPI7	CaMV	Endomembrane system, Vesicle transport	Unknown	Huang *et al.*, 2001
J-domain protein	PMTV	Chaperone, Endomembrane system	Endocytic trafficking the MP to the PM.	Haupt et al., 2005
NtMPIP1	TMV	Chaperone protein	HSP70 co-factor	Shimizu *et al.*, 2009
RNB8	RSV	Chaperone protein	HSP70 co-factor	Lu *et al.*, 2009
cpHSC70-1	AbMV	Chaperone protein	HSP70-like chaperone functions in localisation, translocation	Krenz *et al.*, 2010
DNAJ-like	TSWV	Chaperone protein	Co-factor for HSP-70 associated translocation	Von Bargen *et al.*, 2001
NtCIP	PVY	Chaperone protein	Co-factor for HSP70 mediated intercellular transport	Hofius *et al.*, 2007
NbNACa1	BMV	Chaperone protein	MP translocation	Kaido et al., 2007
RNB5	RSV	Chaperone protein	Chaperoning of the MP-complex through the PD	Lu *et al.*, 2009
Nt-DNAJ-like	TSWV	Chaperone protein	Co-factor for HSP70 mediated intercellular transport	Soellick *et al.*, 2000
HFi22	TBSV	Chaperone protein	Chaperoning of MP/RNA complex through the PD	Desvoyes *et al.*, 2002
NtMBF1a	ToMV	Chaperone protein	Virus modulation of host gene expression	Matsushita *et al.*, 2002
RIO kinase	ToMV, CMV	Phosphorylation	Phosphorylation of MP	Yoshioka *et al.*, 2004

Host Protein	Interacts with MP of:	Category	Predicted function in virus transport	Reference
CK2	PXV, ToMV	Phosphorylation	Stabilisation of MP complexes *in planta*	Modena *et al.*, 2008 Matsushita *et al.*, 2003
TIP1	PVX	Cytoplasmic protein	Recruitment of callose degrading enzymes to increase PD SEL	Fridborg *et al.*, 2003
ANK	TMV	Cytoplasmic protein	Recruitment of callose degrading enzymes to increase PD SEL	Ueki *et al.*, 2010
KELP	ToMV	NCAP	Regulation of host gene expression	Matsushita *et al.*, 2001
Fibrillarin 2	GRV	Nuclear protein	Viral transport complex assembly	Kim *et al.*, 2007
Thaumatin-like protein	CMV	Defence response protein	Unknown	Kim *et al.*, 2005
AtPATL3	TMV, PNRSV	Cytoplasmic protein	Inhibiting the PD localisation of viral complexes	Peiro *et al.*, 2014
AtPATL6	TMV	Cytoplasmic protein	Inhibiting the PD localisation of viral complexes	Peiro *et al.*, 2014
PSI-K	PPV	Component of photosystem 1	Regulation of reactive oxygen species production	Jiminez *et al.*, 2006
2bip	CMV	Unknown	Unknown	Ham *et al.*, 1999
IP-L	ToMV	Unknown	Unknown	Li *et al.*, 2005

Viruses name abbreviations used: GFLV; *Grapevine fanleaf virus, CaMV; Cauliflower mosaic virus, CPMV; Cowpea mosaic virus, TMV; Tobacco mosaic virus, TYLCV; Tomato yellow leaf curl virus, BDMV; Bean dwarf mosaic virus, PLRV; Potato leafroll virus, TCV; Turnip Crinkle virus, BYV; Beet yellows virus, CMV; Cucumber mosaic virus, TSWV; Tomato spotted wilt virus, ORMV; Oilseed rape mosaic virus (formally known as Youcai mosaic virus), CaLCuV; Cabbage leaf curl virus, SLCV; Squash leaf curl virus, PMTV; Potato mop-top virus, RSV; Rice stripe virus, AbMV; Abutilon mosaic virus, BMV; Brome mosaic virus, PVY; Potato virus Y, TBSV; Tomato bushy stunt virus, ToMV; Tomato mosaic virus, PVX; Potato virus X, GRS; Groundnut rosette virus, PNRSV; Prunus necrotic ringspot virus. Other abbreviations used: MP; Movement protein, PD; Plasmodesmata, SEL; Size exclusion limit, HSP; Heat shock protein.*

Chapter two

Is there a role for plasma membrane intrinsic proteins in intercellular movement of plant viruses?

Abstract

Successful infection by plant viruses depends on their interaction with host proteins at various stages of the infection cycle. An *in vitro* association between the movement protein (MP) of *Cowpea mosaic virus* (CPMV) and a plasma membrane intrinsic protein 1 (PIP1) isoform from cowpea (*Vigna unguiculata*) suggested a possible role of PIP1 in tubule-guided intercellular movement of CPMV. We investigated whether this MP/PIP1 association also occurred *in vivo* by localisation of fluorescently labelled MP and PIP1 in *Nicotiana benthamiana*. A PIP1 isoform of *N. benthamiana* localised to the endoplasmic reticulum, while the MP of CPMV accumulated in punctate spots at the periphery of the cell. No co-localisation was observed between the PIP1 and MP fusion proteins. In the presence of over-expressed *N. benthamiana* PIP2, thought to function as chaperone in PIP1 plasma membrane localisation, PIP1 localisation was unchanged.

To investigate the effects of PIP1 silencing on intercellular spread and tubule formation, *N. tabacum* plants in which PIP1 expression was constitutively silenced were infected with GFP-labelled CPMV. Reduced virus spread was observed in leaves of these PIP1-silenced plants at five days post infection. This reduction could not be explained by a direct effect on movement tubule formation, as tubules were formed in equal amounts in both wild type and PIP1-silenced protoplasts.

These results do not rule out a possible interaction between PIP1 isoforms and the MP, however, it seems unlikely that PIP1 isoforms are essential in the tubule formation required for the intercellular spread of CPMV.

Introduction

Intercellular spread of plant viruses to neighbouring uninfected cells involves the action of virus-encoded movement proteins (MPs) that modify the plasmodesmata (PD, plural plasmodesmata,PDs) to allow transport of viral ribonucleoprotein complexes across the cell wall. Modification of PDs is most severe in tubule-guided intercellular movement where MPs form a tubular structure that transfers virions across the PD channel (van Lent and Schmitt-Keichinger, 2006). Assembly of such movement tubules in PDs requires removal of the desmotubule and in some cases dilation of the PD (reviewed by Ritzenthaler and Hoffman, 2007). The native PD is a highly dynamic structure with a size exclusion limit that can actively be adjusted during development or by environmental stimuli (for review see Benitez-Alfonso *et al.,* 2010). Regulation of the size exclusion limit is directed by unknown host proteins which govern the transport of endogenous substrates. However, it is conceivable that such proteins also act in concert with viral MPs to orchestrate intercellular movement of viruses through the PD.

In various studies, host proteins have been identified that associate with viral MPs (see Table 1 in Chapter 1). For several virus/host combinations a dependence on host proteins for successful intercellular transport has been shown (reviewed by Harries and Ding, 2011). The precise role of these host proteins in intercellular transport is unclear for the majority of viruses. Few host proteins, i.e. remorin (Raffaele *et al.*, 2009), calreticulin (Chen *et al.*, 2005), class 1 reversibly glycosylated polypeptides (Zavaliev *et al.*, 2010) and PD-located proteins (PDLPs, Thomas *et al.*, 2008) have been demonstrated to co-localise with MPs at the PD of infected cells. However, only for PDLPs a direct involvement in the cell-to-cell movement of tubule-forming viruses *Grapevine fanleaf virus* and *Cauliflower mosaic virus* was shown (Amari *et al.*, 2010).

In order to identify host proteins that interact with the MP of *Cowpea mosaic virus* (CPMV), which can potentially mediate host modulation by the virus, a column binding assay using immobilized MPs and an enriched plasma membrane (PM) fraction of cowpea (*Vigna unguiculata*) was performed by Carvalho (2003). This assay identified a plasma membrane intrinsic protein 1 (PIP1) with affinity for the MP. Preliminary experiments in which a homologous *Arabidopsis thaliana*

PIP1;4 was co-expressed with the 48 kDa MP of CPMV in cowpea protoplasts showed co-localization of these two proteins in peripheral punctate spots. An interaction was shown by Försters resonance energy transfer (FRET) between the fluorescently labelled MP and AtPIP1;4, although at very low frequency (M. Rolloos, unpublished data). These findings suggest a possible role for PIP1 in the tubule-guided intercellular transport of CPMV.

PIPs are a subgroup in the larger class of membrane intrinsic proteins (MIP). The MIPs are highly conserved proteins which form channels that transport water, gasses and small neutral solutes across membranes in plants, animals and fungi (Zardoya *et al.*, 2001; Gomes *et al.*, 2009). The different groups of MIPs found in plants are categorised based on the membrane they reside in and their structural features (Maurel *et al.*, 2008). PIPs consist of a C-terminal cytoplasmic domain, six trans-membrane domains and a cytoplasmic N-terminal domain (Figure 1A and 1C). PIPs are subdivided in two types, PIP1 and PIP2, this classification is based on the length of the cytoplasmic termini. In the genome of *Arabidopsis thaliana*, five PIP1 isoforms and eight PIP2 isoforms are encoded. The specificity of the PIP membrane channels is dependent on asparagine-proline-alanine (NPA) domains that form the functional selective core of these proteins. PIPs follow Golgi-mediated transport from the endoplasmic reticulum (ER) to the PM, where they assemble as tetrameric channel complexes from functional monomers (Johanson *et al.*, 2001).

As it is known that CPMV MP does not directly interact with the PM (Pouwels *et al.*, 2004), PIP1 could serve as a determinant of membrane identity or as recognition site for MP accumulation which leads to peripheral punctate spot formation and ultimately assembly of movement tubules. A similar role has been found for MIPs in *Cucumber mosaic virus* infection, where an association was shown between viral replication complexes and a tonoplast intrinsic protein, this interaction is thought to facilitate membrane docking of this complex (Kim *et al.*, 2006).

To test whether endogenous PIP1 isoforms interact with MP in a permissive host of CPMV, *Nicotiana benthamiana* (Nb), an *N. benthamiana* PIP1 gene was amplified and expressed as a fluorescent fusion protein. This NbPIP1 was co-expressed with the MP of CPMV in *N. benthamiana* protoplasts to see whether these proteins co-localised and whether an interaction between these proteins

could be established. Furthermore, we tested the effect of PIP1 silencing on tubule formation and intercellular spread in *Nicotiana* plants.

Materials and methods

Plant materials

Cowpea (*V. unguiculata cv. California Blackeye*) plants were grown on Agra-vermiculite (Pull, Rhenen) in a climate chamber under a 16/8 h day/night regime at 28/20°C respectively. *N. benthamiana* (Nb) and *N. tabacum* (Nt) Samsun NN (wild type and PIP1 silenced) plants were grown on soil at 24°C in a greenhouse.

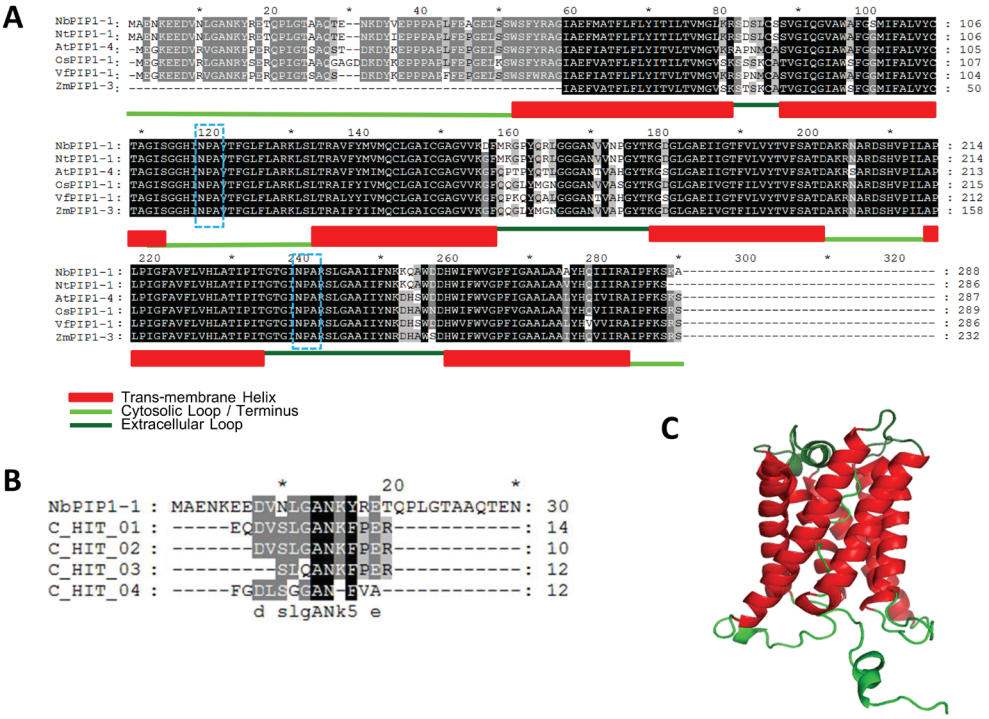

Figure 1: Amino acid alignments and structural predictions of NbPIP1-1.
A) Alignment of NbPIP1-1 to sequence-verified PIP1 isoforms of *Nicotiana tabacum* (Nt), *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Vicia faba* (Vf), and *Zea mays* (Zm), with secondary structure prediction of transmembrane helices (red boxes) and connecting loops (green lines) and characteristic PIP1 NPA domains (blue dashed boxed) indicated. **B)** Alignment of fragments identified by Carvalho (2003) to the N-terminus of the cloned NbPIP1-1. **C)** Predicted three-dimensional protein structure of NbPIP1-1, illustrating the orientation of the 6 transmembrane domains and the long N-terminal cytosolic domain (colours correspond to predictions in A).

The PIP1 silenced *N. tabacum* Samsun NN plants were a kind gift from Dr. Franka Siefritz and have been described as Nt^{-AQP1} in Siefritz *et al.* (2002).

Viruses

CPMV was used to infect wild type and PIP1 silenced *N. tabacum* Samsun NN protoplasts. Mechanical infection of *N. tabacum* plants was done with recombinant CPMV-M19GFP2A, in which the GFP gene is inserted in RNA2 between the MP and the large capsid protein (LCP) as described by Gopinath *et al.* (2000). This recombinant virus expresses GFP partly as free protein and partly fused to the N-terminus of LCP.

Protoplast isolation, transfection, and infection

Cowpea mesophyll protoplasts were isolated from leaves as described by van Bokhoven *et al.* (1993). *Nicotiana* mesophyll protoplasts were prepared using the same protocol with the exception that 3 cm long leaves were repeatedly cut from the midvein outwards, resulting in a featherlike leaf, to allow enzymatic digestion of the cell walls. *Arabidopsis thaliana* protoplasts were isolated as described by Sheen (2002). CPMV infection (10 µg per 10^6 protoplasts) and DNA transfections (5 µg per 10^5 protoplasts) of protoplast, using polyethylene glycol (Mw 6,000), were performed as described van Bokhoven *et al.* (1993).

Cloning and generation of fluorescent NbPIP1-1 fusion proteins

RNA of *N. benthamiana* plants was isolated using Trizol (Invitrogen) and cDNA was generated using a poly-adenylation site binding primer (oligo-dT) and *Avian myeloblastosis virus* reverse transcriptase as described by the manufacturer (Promega). The cDNA was used as template for PCR amplification of the *N. benthamiana* PIP1 coding sequence using NtPIP1-1 and NtPIP1-2 primers described by Bots *et al.* (2005). An 867 base pair (bp) product was amplified (at an annealing temperature of 58°C) and its sequence was verified to encode a *N. benthamiana* PIP1 gene (NbPIP1-1). BglII/NcoI restriction sites (underlined) in forward (5′ GCAGATCTGAAATGGCAGAAAACAAAGAAGAAG 3′) and reverse primer (3′ GCCCATGGCAGCCTTGCTCTTGAATGGA 5′) were used to generate C-terminal fusions of NbPIP1-1 (minus stop codon) to CFP and YFP in the pMON-MP-CFP/YFP vector backbones (Pouwels *et al.*, 2003). The same

approach was followed for the amplification of the NbPIP2-1 gene (855 bp product) with the use of a start codon (bold) encompassing forward primer (5' CGAAGATCT**ATG**TCAAAGGACGTGATTGAAG 3') and a C-terminal reverse primer (3' CGACCATGGCGTTGGTTGGGTTACTGCG 5') also containing BglII/ NcoI restriction sites (underlined) respectively.

Immunofluorescence studies

Protoplasts were fixed on 0.05% poly-L-lysine (Mw 353,000; Sigma) coated Menzel-superfrost slides (Thermo scientific) and permeabilised by treatment with 96% ethanol for 30 min to reduces chlorophyll auto-fluorescence. After washing 3 times 5 min in phosphate buffered saline (PBS, pH 7.4), protoplasts were blocked by incubation in 1% bovine serum albumin (BSA) in PBS at 37°C for 60 min. Protoplasts were incubated for one h with anti-58K antibody (Kasteel *et al.*, 1996) diluted 1:400 in 1% BSA in PBS at 37°C, washed and incubated with goat anti-rabbit Alexa488 conjugate (diluted 1:500; Molecular probes). Protoplasts were then washed and embedded in Citifluor (Citifluor Ltd) anti-fading agent.

Microscopical analysis

Fluorescence microscopy studies were done with a Zeiss LSM 510-META confocal microscope operated through Axiovision software. Multi-coloured fluorescent protoplasts were analysed using the multichannel acquisition mode to prevent fluorescent bleed-through of fluorophores. For GFP excitation an Argon-laser (30mW) was used at 488nm, emission was measured between 505-570nm, YFP was excited using a 514nm laser line and emission was detected at 530-600nm. CFP was excited with a 458nm laser line and emission was measured between 470 and 500nm. For PM labelling the lipophilic dye FM 4-64 (Molecular probes) was added to protoplasts at a final concentration of 0.66 mg/ml and imaged using 514nm laser line excitation combined with 650nm long pass emission filters. Measurement of infection foci on CPMV-GFP-infected *N. tabacum* leaves was done using a binocular microscope (Leica MZFL3) suited for GFP imaging and a TCC-1.4ChICE camera (Tucsen imaging, China). The size of infection foci was determined by manual tracing of the contour using the polygon selection tool of ImageJ (version 1.48d, Schneider *et al.*, 2012).

Significant differences between the size of infection foci in the different genetic

backgrounds were tested as follows. Data were checked for normality and equality of variance using Kolmagorov-Smirnov and Levene's tests respectively. Square root transformed data were normally distributed but not of equal variance, therefore, an independent samples t-test was used to show that the foci size difference between wild type and PIP1 silenced *N. tabacum* plants was significant with a two-tail significance value of $P<0.01$.

Protein analysis

Western blot analysis was done using 0.1 g leaf material of *N. benthamiana* and *V. unguiqulata*. Leaf material was collected and plunge frozen in liquid nitrogen, frozen material was ground to powder and submerged in 200 µl denaturation buffer with 1.5% (v/v) beta-mercaptoethanol (Laemmli, 1970). Samples were heated to 95°C for five min, cooled on ice and centrifuged for 1 min. at 15,000 *g* prior to loading 25µl of supernatant on a 12% bis-acrylamide gel for protein size separation. Proteins were semi-dry blotted onto Immobilon-P membrane (Millipore). Blots were developed using primary anti-PIP1 antibodies diluted 1:1000 in PBS (kindly provided by Prof. dr. Anton Schäffner and described in Kammerloher *et al.,* 1994) and secondary goat anti-chicken alkaline phosphatase conjugates (Sigma) used according to manufacturers instructions. Microsomal fractions were isolated as described by Mahdieh and Mostajeran (2009) using 10 g of fresh leaf material as input.

Software tools used

Protein sequence alignments were done using Genedoc software (Nicholas *et al.*, 1997). Secondary structures were predicted using the transmembrane helix prediction server of the technical university of Denmark (TMHMM 2.0c, The Centre for Biological Sequence Analysis TDU, Denmark). Three dimensional protein predictions were obtained through the Phyre^2 server (http://www.sbg.bio.ic.ac.uk/phyre2/; Kelley and Sternberg, 2009) with visualisation and colour coding done using the PyMOL software (DeLano Scientific LLC, USA). Statistical tests were performed using IMB SPSS statistics software package version 21.

Results

Subcellular localisation of *N. benthamiana* PIP1

N. benthamiana was chosen as experimental host because CPMV replicates and spreads efficiently in this plant species and it permits reverse genetic experiments. A PIP1 gene from *N. benthamiana* was cloned from cDNA using primers based on a closely related *N. tabacum* PIP1. The amplified coding sequence, which will be referred to as NbPIP1-1 henceforth, showed 85% homology to the *A. thaliana* PIP1;4 and 97% to the PIP1-1 gene of *N. tabacum*. (Figure 1A). NbPIP1-1 also contained the hallmark features of the PIP1 subgroup, i.e. a long N-terminal cytosolic domain, six predicted transmembrane helixes and two NPA domains (Figure 1A, and 1C). The peptides that identified the MP binding protein in the study of Carvalho (2003) also aligned to NbPIP1-1 sequence (Figure 1B).

To determine the cellular location of the NbPIP1-1 protein, C-terminal fusions were made to both CFP and YFP. Expression of the NbPIP1-1 fusion proteins in protoplasts resulted in a clear fluorescent signal, indicating that the fusion protein was expressed (Figure 2A, and 2J). The majority of fluorescent signal was observed in internal structures of the protoplast and little signal originated from the periphery of the protoplast (see overview in figure 2A and zoomed image in figure 2E). The localization of NbPIP1-1, however, did not resemble the localization of arabidopsis PIP1 (compare Figure 2A and Figure 2D) nor did it localise in a similar fashion as the PM marker FM 4-64 (compare Figure 2A and Figure 2H). Indicating that the NbPIP1-1 fusion protein did not localise to the PM.

Co-localisation studies of NbPIP1-1 with CPMV MP and NbPIP2-1

Co-expression of YFP-labelled NbPIP1-1 with CFP-labelled CPMV MP in *N. benthamiana* protoplasts did not show co-localisation of the two proteins (Figure 2A-C). MP-CFP was found as punctate spots at the periphery of the cell and in tubules emanating from the protoplasts surface, whereas the NbPIP1-1-YFP was primarily confined to internal structures (compare Figures 2A and 2B, and their overlay in 2C). The low amount of NbPIP1-1-YFP that is found at the periphery of the cell did not co-localise with the peripheral punctate MP spots (indicated by arrows in Figures 2E-G). The localisation of a green fluorescent ER marker (GFP-

HDEL) showed striking similarity to the localisation of NbPIP1-1, indicating that this protein could also be associated with this membrane (compare Figures 2A and 2L).

ER localisation of NbPIP1-1 was unexpected and could be due to deficiencies in export from the ER to the PM. In maize (*Zea mays*) mesophyll protoplasts transport of overexpressed ZmPIP1-YFP from the ER to the PM required hetero-dimerization of ZmPIP1 with overexpressed ZmPIP2 isoforms (Zelazny *et al.*, 2007). Based on these findings in maize and a reciprocal set-up in *A. thaliana* (Sorieul *et al.*, 2011), a *N. benthamiana* PIP2 gene (NbPIP2-1) was cloned and C-terminally fused to CFP and YFP. Co-transformation of protoplasts with both NbPIP1-1 and NbPIP2-1 fluorescent constructs (under the same 35S promoter), however, did not cause relocation of NbPIP1-1 to the PM. A high degree of co-localisation was, nonetheless, observed between the NbPIP1-1 and NbPIP2-1 fluorescent fusion proteins in the ER of these cells (Figure 2I-2K). Because NbPIP1-1 exclusively localised to the ER, even when co-expressed with NbPIP2 fusion proteins, no co-localisation of the MP and PIP1 fusion proteins could be observed at the periphery of the cell.

Immunological detection of NbPIP1-1

To test whether endogenous NbPIP1 isoforms resides in the PM and co-localizes with CPMV MP, an immunolabelling was done using an antibody that was previously shown to recognise *N.tabacum* PIP1 proteins (Mahdieh and Mostajeran, 2009). The epitope recognised by this antibody, the 42 N-terminal amino acids of (At)PIP1 (Kammerloher *et al.*, 1994), is nearly identical between NtPIP1-1 and NbPIP1-1 (Figure 1A). Despite the high similarity in epitopes, this anti-PIP1 antibody was unable to recognise NbPIP1 proteins in an immunolabelling of *N. benthamiana* protoplasts (data not shown).

In a western blot analysis of proteins from *N. benthamiana* leaf tissue, protein bands of 38kDa and 23 kDa were detected by the anti-PIP1 antibody (indicated by * in Figure 3A). The size of these protein bands did not correspond to the predicted size of the NbPIP1-1 monomer (30.9 kDa, ExPASy.org, Compute pI/Mw; Wilkins *et al.*, 1999) or the possibly present NbPIP1-1 dimer, even if predicted phosphorylation of the serine at position 130 (Johanson *et al.*, 2001) is taken into account. PIP1 monomer and dimer bands observed in the microsomal

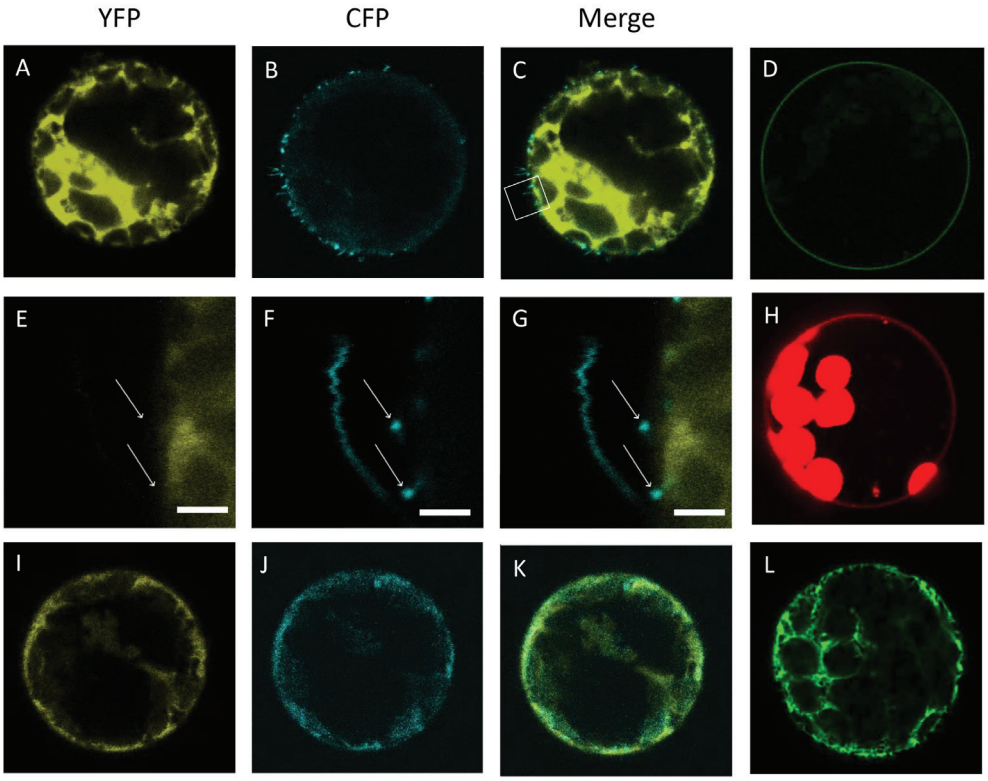

Figure 2: Confocal images showing localisation of fluorescent fusion proteins in *N. benthamiana* protoplasts.
A-C) Localisation of NbPIP1-1-YFP and CPMV MP-CFP, the merged image shows that NbPIP1-1-YFP localises mainly internally and MP-CFP is found in peripheral punctate spots and short tubules. **D)** PM localisation of arabidopsis PIP1;2-GFP in arabidopsis protoplasts. **E-G)** Detailed image of an NbPIP1-1 and MP-CFP transfected cell (magnified area indicated by box in C). NbPIP1-1 is not localised to the PM nor does it localise with MP-CFP (arrows). **H)** FM 4-64 dye staining plasma membrane (PM), chloroplast auto-fluorescence can also be observed. **I-K)** Co-expression of NbPIP2-1-YFP and NbPIP1-1-CFP co-localisation of the two proteins is apparent, and similar to the ER localisation illustrated in L. **L)** Localisation of ER retained GFP-HDEL. Protoplast diameter ranged from 25-39 µm, scale bar in E-G is 2 µm.

fraction of root material of *N. tabacum* (32 and 64 kDa respectively, Mahdieh and Mostajeran, 2009) could not be detected on western blot, when the described protein isolation protocol was applied to *N. benthamiana* leaf material (Figure 3B). These data collectively suggest that the anti-PIP1 antibody does not react with PIP1 isoforms from *N. benthamiana* leaves and hence is not suitable for localization studies in this host.

Silencing PIP1 genes hampers intercellular spread, but not movement tubule formation, during CPMV infection

PIP1 silenced *N. tabacum* plants (Siefritz *et al.*, 2002) were infected with CPMV-GFP to test whether silencing of *Nicotiana* PIP1 genes had an effect on local virus spread. These tobacco plants constitutively express an antisense PIP1 gene which down regulates the expression of several PIP1 isoforms (Siefritz *et al.*, 2002). Multicellular fluorescent infection foci, observed at 5 days post infection, were smaller in PIP1 silenced plants than in non-transgenic wild type plants (Figure 4A). This indicated that down regulation of PIP1 genes resulted in a small but significant reduction in the speed of intercellular spread of the virus from the initial infected cell (Figure 4B).

Protoplast from these PIP1 silenced plants infected with CPMV showed the formation of movement tubules at the protoplast surface. No difference was

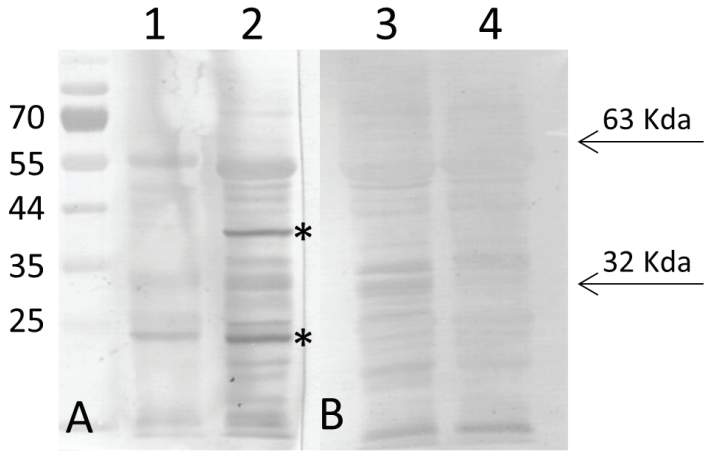

Figure 3: Western blot analysis of leaf homogenates probed with anti-PIP1 antibody. **A)** Crude leaf extracts of cowpea (lane 1) and *N. benthamiana* (lane 2). **B)** microsomal fractions of healthy (lane 3) and CPMV infected (lane 4) *N. benthamiana* leaves. In lane 3 and 4 the expected (based on Mahdieh and Mostajeran, 2009) 32 and 64 kDa PIP1 monomer and dimer bands could not be observed. * indicates the a-specific bands detected by the anti-PIP1 antibody in *N. benthamiana*.

observed in timeframe, morphology or abundance of tubules formed on protoplasts from either wild type or PIP1-silenced *N. tabacum* leaves (Figure 4c). This suggests that the silencing of PIP1 genes has no effect on the formation of movement tubules.

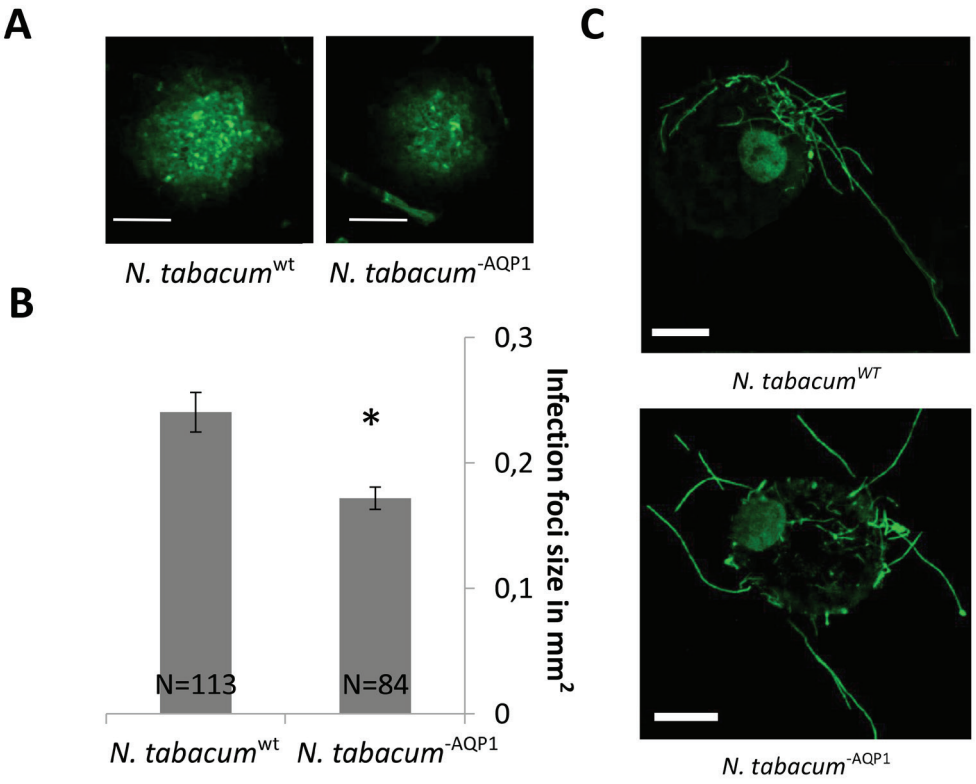

Figure 4 Viral spread and tubule formation in wild type and PIP1-silenced (-aqp) *N. tabacum.*
A) Size and shape of fluorescent infection foci and **B)** average size of the foci in the two *N. tabacum* genotypes at 5 days after CPMV-GFP infection. Error bars indicate standard error, N= number of foci measured in three individual experiments, * indicates a significant reduction in foci size in the PIP1 silenced plants (T-test, P<0.01). **C)** immunofluorescent detection of CPMV MP in infected protoplasts of wild type and PIP1-silenced *N. tabacum*. Scale bar in A is 250 µm, in C is 5 µm.

Discussion

Plasma membrane intrinsic protein 1 (PIP1) was identified in an *in vitro* binding assay as a candidate host protein for interaction with CPMV MP in a previous study by Carvalho (2003). We have further investigated the putative role of this class of host proteins in tubule-guided intercellular movement. A homologous PIP1 gene from the experimental host *N. benthamiana* was identified (NbPIP1-1), but co-localisation studies with CPMV MP and NbPIP1-1 in protoplasts revealed a distinct cellular localisation of these proteins, hence a role for PIP1 in CPMV movement could not be confirmed.

The *N. benthamiana* PIP1 gene amplification from cDNA produced a single product that showed high amino acid sequence homology to various known PIP1 sequences (Figure 1A) and was predicted to be localised to the PM (WoLF PSORT, Horton *et al.*, 2007). This indicated that the amplified gene from *N. benthamiana* codes for a PIP1 isoform. Alignment of peptides from the cowpea PIP1 to the protein sequence of NbPIP1-1, indicated that this *N. benthamiana* PIP1 was homologous to the PIP1 isoform found by Carvalho (2003).

However, the location of fluorescently labelled NbPIP1-1 expressed in *N. benthamiana* protoplasts appeared to be mostly intracellular with a pattern resembling the localisation of ER-retained GFP-HDEL. This observation contradicts the presumption that PIP1 isoforms are found in the PM and only transiently reside in the ER during synthesis. On the contrary, expression of an arabidopsis PIP1-GFP fusion showed the expected localization of the protein at the PM (compare Figure 2A with Figures 2D)(Boursiac et al., 2005). The localisation of the NbPIP1-1 fusion proteins to the ER cannot be explained by the presence of known retention signals in the amino acid sequence (Gomes *et al.*, 2009), but might be an artefact caused by overexpression. ER retention due to incorrect folding seems unlikely as the physicochemical properties (polarity, size and charge) of NbPIP1-1 are very similar to PIP1 members found in *N. tabacum*, arabidopsis and rice (*Oryza sativa*) (Figure 1A and 1 C). Distortion of PIP1 localisation by the C-terminal fluorescent fusion protein is not very likely as these fusions have been proven functional for other PIP1 proteins (Figure 2D, Boursiac *et al.*, 2005; Zelazny *et al.*, 2007; Sorieul *et al.*, 2011), but a pleiotropic effect cannot be ruled out.

Studies of Zelazny and colleagues (2007) in maize protoplasts showed that correct PIP1 localisation is dependent on chaperone activity of PIP2 proteins when overexpressed in their native environment. Because of this observation and the known hetero-oligomerisation of *Nicotiana* PIP isoforms (Otto *et al.*, 2010), fluorescent fusions of a NbPIP2 were generated and co-expressed with the PIP1 fusion proteins. In *N. benthamiana* protoplasts the combined overexpression of PIP2 did not cause any notable alterations in the localisation of NbPIP1-1. The NbPIP1-1 proteins remained localised to the ER where high levels of co-localisation with the NbPIP2-1 fusion protein were observed. The reason for ER localisation of these two PIPs is unclear, the PIP2 might not be compatible as a chaperone of the PIP1 or might not be able to exit the ER itself. The latter possibility could be due to the absence of a di-acidic motive required for AtPIP2;1 ER export (Sorieul *et al.*, 2011). This is, however, speculative as various PIP2 isoforms (in both arabidopsis and other plants) lack this motif (Figure 5) but do localise to the PM.

The fact that the NbPIP1-1 does not exhibit the expected PM localisation, prevents investigation of a possible association of PIP1 with the MP of CPMV, as observed in *in vitro* experiments by Carvalho (2003). Although our results do not indicate a PIP1–MP association *in vivo*, a possible interaction between a PIP1 isoform and the CPMV MP cannot be excluded. In arabidopsis five highly conserved and possibly functionally redundant PIP1 isoforms are encoded (Gomes *et al.*, 2009). The number of PIP1 genes in *N. benthamiana* is unknown, but it is likely that this genome also contains multiple PIP1 isoforms. A BLAST search of the *N. benthamiana* genome (Bombarely *et al.*, 2012), using a query of the five prime 240 (translated) nucleotides of NbPIP1-1, resulted in 9 predicted protein hits, of which five had corresponding predicted cDNAs in the translated database. These database hits showed more than 81% sequence similarity to the NbPIP1-1 gene (Figure 6) and illustrate the likelihood of multiple PIP1 members in the *N. benthamiana* genome. Because only one PIP1 gene was amplified and experimentally tested in this study we cannot generalize our finding to all NbPIP1 isoforms. It is possible that another PIP1 isoform does interact with the MP of CPMV at the PM. Testing this hypothesis would require identification, amplification and fluorescent labelling of all PIP1 members of *N. benthamiana*, which is technically challenging as annotation of the *N. benthamiana* genome is

incomplete.

As an alternative approach to test whether PIP1 isoforms affect CPMV tubule-guided transport, the spread of a GFP labelled CPMV was recorded in a *Nicotiana* host in which multiple PIP1 genes were silenced (Siefritz *et al.*, 2002). The significant difference in size of the infection foci five days after inoculation illustrates that down regulation of multiple PIP1 genes slows intercellular virus spread in this host. Because the PIP1 proteins were suspected to associate with the MP of CPMV, the functionality of the MP was tested by transfection to protoplasts prepared from this silenced host. Tubule formation is the ultimate manifestation of MP functionality as it requires concerted action between MP and host factors. The silencing of PIP1 isoforms did not hamper tubule formation. The number of tubules, their morphology and the timeframe in which tubules appeared was similar in both genotypes. The fact that MP function is unaffected when the expression of multiple PIP1 genes is downregulated questions the biological relevance of the observed PIP1 binding to immobilised MP in an *in vitro* situation by Carvalho (2003) and suggests this might have been a false positive interaction. Even though our localisation experiments do not conclusively answer the question whether PIP1 isoforms interact with CPMV MP during infection, the unaltered tubule formation in a PIP1 silenced background suggests that if such an interaction occurs during infection it does not notably hamper or evidently aid the functioning of the MP of CPMV.

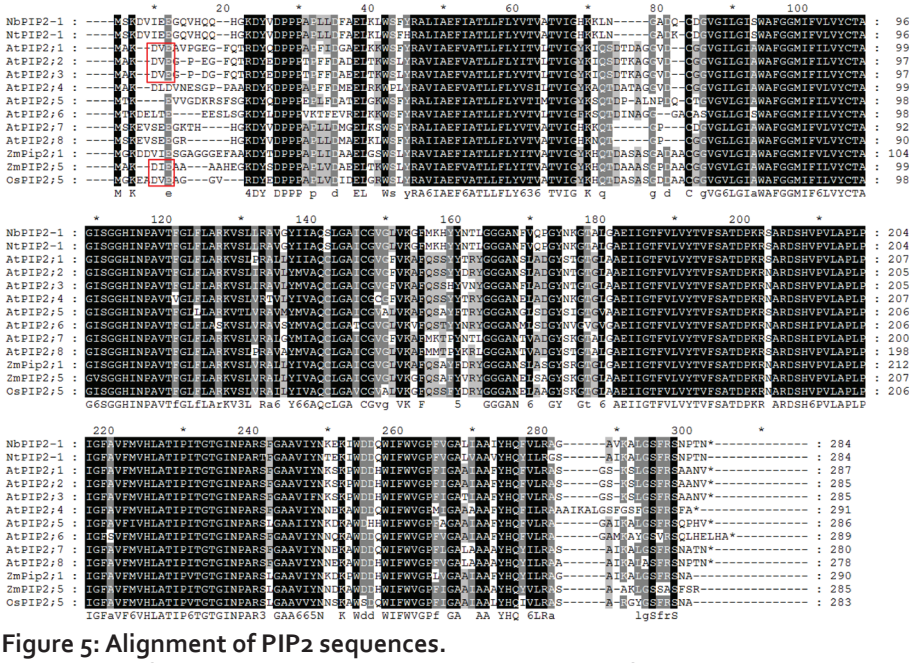

Figure 5: Alignment of PIP2 sequences.
Alignment of *N.benthamiana* PIP2-1 to PIP2 genes of *Nicotiana tabacum* (Nt), *Arabidopsis thaliana* (At), *Zea mays* (Zm) and *Oryza sativa* (Os). Red boxes indicate the di-acidic motive (D^4xE^6) required for AtPIP2;1 ER export.

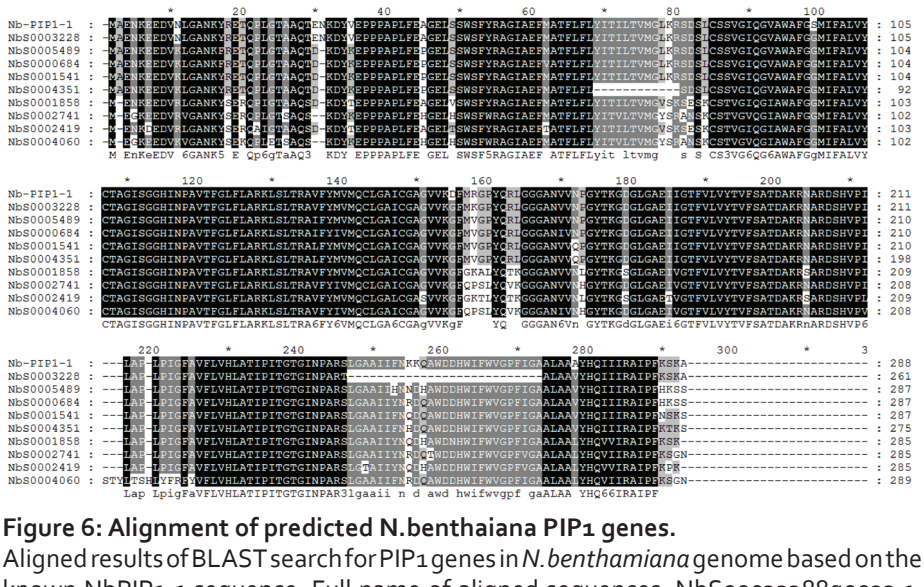

Figure 6: Alignment of predicted N.benthaiana PIP1 genes.
Aligned results of BLAST search for PIP1 genes in *N.benthamiana* genome based on the known NbPIP1-1 sequence. Full name of aligned sequences; NbS00032288g0022.1, NbS00054890g0003.1, NbS00006841g0003.1, NbS00015419g0005.1, NbS00043512g0006.1, NbS00018582g0001.1, NbS00027416g0007.1, NbS00024194g0106.1, NbS00040601g0014.1.
For underlined sequences a cDNA sequence is available.

Chapter three

Proteomic analysis of the plasma membrane-movement tubule complex of *Cowpea mosaic virus*.

To be re-submitted as:
P.W. den Hollander, P. de Sousa Geraldino Duarte, H. Bloksma, S. Boeren and J.W.M. van Lent.
Proteomic analysis of the plasma membrane-movement tubule complex of Cowpea mosaic virus.

Abstract

Cowpea mosaic virus forms tubules constructed from the movement protein (MP) in plasmodesmata to achieve cell-to-cell movement of its virions. Furthermore, similar tubules are formed protruding from the surface of infected protoplasts. In both situations the tubule is delineated by the plasma membrane (PM). We have isolated the PM-tubule complexes from protoplasts by immunoprecipitation and analysed their protein content by tandem mass-spectrometry to identify host proteins with affinity for the movement tubule. Seven host proteins were found to be abundantly present in the PM-tubule complex. These included molecular chaperonins and an AAA-protein. Members of both protein families have been implicated in establishment of systemic infection. The potential role of these proteins in tubule-guided cell-cell transport is discussed.

Introduction

Cell-to-cell movement of plant viruses occurs through plasmodesmata (singular plasmodesma, PD) by different mechanisms depending on virus family and genus (recently reviewed by Schoelz *et al.*, 2011). Movement of an infectious viral entity is only achieved after modification of the PD by actions of virally encoded movement proteins (MPs). The MPs of viruses that traffic their genome between cells as mature virus particles alter the PD architecture drastically. The desmotubule is removed from the plasmodesmal pore and replaced by a movement tubule assembled from MP, which carries virions to adjacent cells (Waigmann *et al.*, 2004; van Lent and Schmitt-Keichinger, 2006; Ritzenthaler and Hofmann, 2007), a complex process that probably relies on host proteins.

Cowpea mosaic virus (CPMV) and *Grapevine fanleaf virus* (GFLV) are examples of viruses that transport their virion through PD via a movement tubule (van Lent *et al.*, 1990; Ritzenthaler *et al.*, 1995). For CPMV the 48 kDa MP is the only viral protein required for tubule formation as was illustrated by transient expression of this protein in plant protoplasts (Wellink *et al.*, 1993). In plant tissue, movement tubules are formed in PD, in close contact with the plasma membrane (PM). In protoplasts and even in insect cells (Kasteel *et al.*, 1996), in the absence of PD, tubules are anchored at the PM and grow into the culture medium tightly engulfed by the expanding PM. These observations suggest that the PM is an important host structure for tubule assembly and that host proteins involved in anchoring and assembly of the tubule are conserved between taxonomical kingdoms.

Several host proteins have been reported to be involved in plant virus movement (see reviews by Niehl and Heinlein, 2011 and Schoelz *et al.*, 2011). More specifically, remorin (Raffaele *et al.*, 2009), calreticulin (Chen *et al.*, 2005), class 1 reversibly glycosylated polypeptides (Zavaliev *et al.*, 2010) and PD located proteins (PDLPs, Thomas *et al.*, 2008) co-localize with MPs at the PD of infected cells. Of these, PDLPs are the only proteins shown to be required for successful cell-cell movement, in which they directly interact with the MPs at the base of the tubule-PD complex (Amari *et al.*, 2010). For CPMV Pouwels *et al.* (2004) showed that the interaction between the MP and the PM is not direct but probably involves PM-associated host proteins. To identify such proteins we performed a tandem mass-spectrometry analysis of proteins present in the PM-tubule

complex isolated from CPMV-infected cowpea protoplasts.

Materials and methods

Isolation of movement tubules from infected protoplasts

Cowpea (*Vigna unguiculata* var. California blackeye) protoplasts were prepared and inoculated with 10 µg of purified CPMV per 10^6 protoplasts using polyethylene glycol (Mw 6,000; van Bokhoven *et al.*, 1993). At 40 h post infection, tubules were sheared from the protoplast surface by vortexing the suspension at 1,600 rpm for 1 min. Cells and large fragments were pelleted by centrifugation for 5 min at 40 *g*. The supernatant was centrifuged at 15,000 *g* for 15 min to sediment the tubules. The sediment was re-suspended in 50 µl of a microtubule-stabilizing buffer (PEM buffer, Scheel and Kreis, 1998) and the suspension was cleared by short centrifugation (10 sec at 15,000 *g*). The clear supernatant contained the PM-tubule complex and was used as input for immunoprecipitation.

Immuno-precipitation

To allow recognition of the movement tubule by anti-MP antibody, the PM surrounding the tubule was permeabilised in 0.5% (v/v) Nonidet P-40 (in PEM buffer) for 30 min. Tubules were purified by immunoprecipitation using protein A-coated magnetic beads (Dynabeads, Invitrogen) to which antibodies were cross-linked using Bis(sulfosuccinimidyl)suberate (Sigma) according to the manufacturers protocol. For the isolation of PM-tubule complexes, beads were cross-linked with anti-MP antibodies and for negative control experiments, beads were cross-linked with rabbit pre-immune serum. These beads were incubated with the tubule suspension overnight at 4°C under constant mild agitation. The beads were then washed three times in PEM buffer supplemented with 0.02% (v/v) Nonidet P-40 and transferred to a clean tube to avoid co-elution of proteins bound to the tube wall. Proteins were eluted from the beads by heating to 70°C for 10 min in SDS sample buffer containing β-mercaptoethanol (Laemmli, 1970).

Immunolocalisation

Immunolocalisation of MP in CPMV-infected protoplasts was done using a polyclonal antiserum against the MP (anti-58K, Kasteel *et al.*, 1996) as described previously (van Lent *et al.*, 1991).

Electron microscopy

The tubular fractions that served as input for immuno-precipitation were negatively stained with 1% phosphotungstic acid as described (van Lent *et al.*, 1991) and inspected using a JEM1011 (Jeol) microscope equipped with a Keenview camera (SIS, Olympus).

Western Blotting

Immunoprecipitation eluates were diluted (1:1) in denaturation buffer with 1.5% (v/v) beta-mercaptoethanol (Laemmli, 1970). Samples were heated to 95°C for five min, cooled on ice and centrifuged for 1 min at 15,000 *g* prior to loading on a 12% bis-acrylamide gel, for protein size separation. Proteins were semi-dry blotted onto Immobilon-P membrane (Millipore). Blots were developed using primary anti-58K or anti-CPMV antibodies (Wellink *et al.*, 1987) diluted 1:1000 in PBS, secondary alkaline phosphatase conjugated antibodies (Sigma) were used according to manufacturers instructions.

Mass spectrometry analysis and peptide annotation

Proteins eluted from the beads in the different treatments were were size separated in individual lanes by SDS-PAGE in 12% acrylamide gels (Laemmli, 1970). Proteins were visualized using Oriole stain (Invitrogen) and cut from the gel. Proteins were in-gel digested and peptides were extracted from the gel as described by Kariithi *et al.* (2011). Peptides were separated by reversed phase nano liquid chromatography (LC) prior to Fourier transform mass spectra (MS) measurements as described (Lu *et al.*, 2011). LC-MS runs with all tandem MS spectra obtained were analysed with MaxQuant (Cox and Mann, 2008) using default settings for the Andromeda search engine (1% False Discovery rates both on peptide and protein level) (Cox *et al.*, 2011) with extra variable modifications set for de-amidation of asparagine and glutamine. Because a limited number of cowpea protein sequences is known (Uniprot.org database) a custom made protein database was used to search the tandem MS data against. This custom database contained all known proteins from plants of the order *Fabales*, which includes cowpea and sequenced organisms such as soybean (*Glycine max*) and barrel medic (*Medicago truncatula*) (145,076 proteins, downloaded from www.uniprot.org, December 2012). Furthermore, MP interacting host proteins known

from literature were included in the database (Table 1). Finally, all CPMV encoded proteins were added to this database. A database containing sequences of common contaminants like bovine serum albumin, trypsin and human keratins was used to carry out the first search. For protein quantification the "label-free quantification" (LFQ) and "match between runs" options were enabled, the use of de-amidated peptides was allowed and all other quantification settings were kept default.

The MaxQuant output (protein groups result) was additionally filtered based on four criteria: (i) a minimum of two peptides should align to a protein, (ii) at least one of these peptides should be unmodified, (iii) at least one of these peptides should be unique for this protein group and (iv) proteins are present in both isolations. Reversed hits were deleted from the MaxQuant results as well as hits showing a LFQ value of zero for both sample and control. Zero values for one of the two log LFQ columns (representing one of the two isolation experiments) were replaced by a value of 4.9 (slightly below the lowest measured value) to allow ratio calculations.

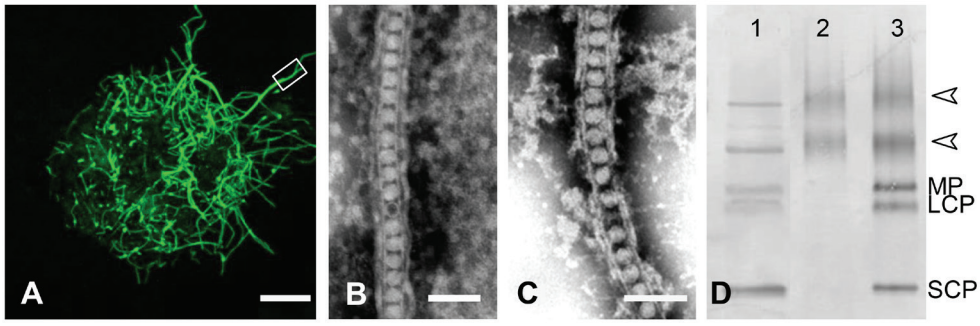

Figure 1: Analysis of CPMV plasma membrane-movement tubule complex.
A) Immunofluorescent image of a CPMV infected cowpea protoplast showing massive tubule production. **B,C)** Transmission electron micrographs of isolated tubule (boxed in a) before and after treatment with Nonidet P-40 respectively. **D)** Western blot of CPMV-infected cowpea protoplasts proteins treated with anti-MP and anti-CPMV antibodies showing the MP (48kDa),large (37kDa) and small coat protein (23kDa) (LCP and SCP) presence in the tubule suspension used as input for immunoprecipitation (lane 1), the supernatant containing unbound proteins after incubation with magnetic beads (lane 2) and the elute from the beads (lane 3). Protein smears caused by antibodies eluted from the beads are indicated by arrowheads. Scale bars 5 µm(a) and 100 nm(b,c).

Results

Isolation of plasma membrane-movement tubule complexes

The infection of cowpea protoplasts was confirmed by immunofluorescent labelling of the MP, this labelling also enabled quantification of tubule formation (Figure 1A). Batches of protoplast with infection percentages above 85% and which showed ample tubule formation were used to isolate the PM-tubule complexes. The presence of the PM-tubule complex in the isolated tubule fraction was verified by electron microscopy (Figure 1B) prior to further purification of the complex by immunoprecipitation. Electron microscopic analysis showed that treatment of the isolated tubules with a mild detergent partially disrupted the PM, which would allow antibodies to bind to the MP (Figure 1C).

The fraction containing isolated tubules was further purified by immunoprecipitation using anti-MP antibodies. Western blot analysis of the immunoprecipitation eluate showed the presence of all expected viral proteins,

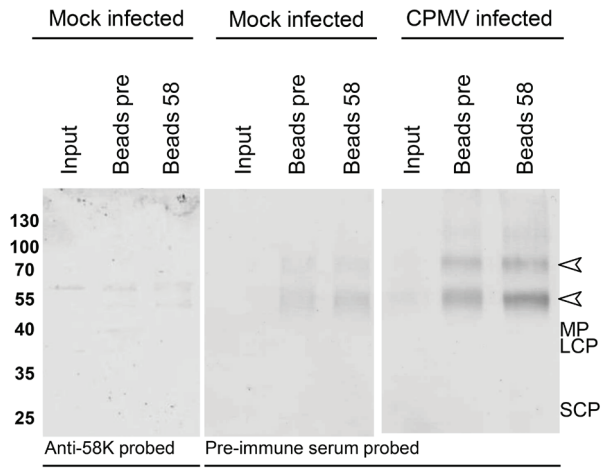

Figure 2: Antibody specificity.

Western blots of control experiments show that host- and viral proteins do not specifically bind the beads used in the immunoprecipitation assays. *Left blot:* immunoprecipitation of mock-infected protoplasts using beads coated with pre-immune serum (Beads pre) or anti-58K antibodies (Beads 58) and blot probed with anti-58K antiserum. *Middle and right blot:* similar immunoprecipitation of mock-infected and CPMV-infected protoplasts and blots probed with pre-immune serum. Protein smear at ±55kDa and 90 kDa represent antibodies eluted from the beads (indicated by arrowheads). Blots incubated with alkaline phosphate substrate at least as long as blot shown in Figure 1D.

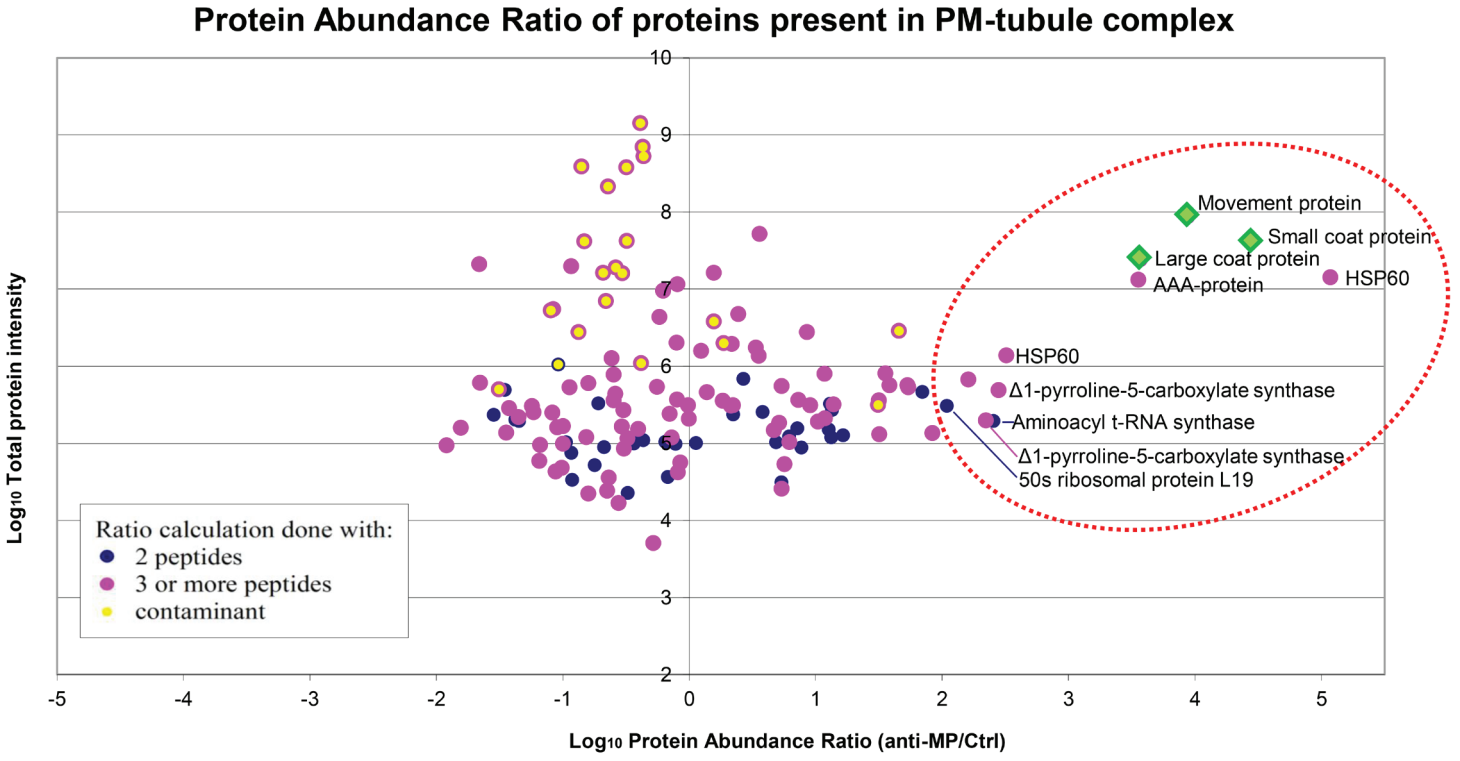

Figure 3: Mass spectrometric analysis of the PM-tubule complex isolated from infected cowpea protoplasts.
The Y-axis shows the relative protein abundance (total iBAQ intensity) as logaritmic values, and the X-axis shows the log(10) of the abundance ratio of proteins precipitated with the anti-MP antiserum over proteins precipitated with pre-immune serum. Proteins that are at least 100-fold enriched (encircled) are considered candidate components of the PM-movement tubule complex. Contaminants are shown in yellow and viral proteins in green.

i.e. the MP and two capsid proteins (Figure 1D). As a negative control the same immunoprecipitation protocol was performed using the same isolated tubule suspensions as input, but with beads coated with pre-immune serum, which do not specifically bind any virus or host proteins (Figure 2). Interactions of proteins with the pre-immune serum-coated beads can thus be classified as non-specific / false positive. For the analysis of the tubular proteome we used eluted proteins from two tubule isolation experiments, and their respective controls, which were size separated in individual lanes by SDS-PAGE. Proteins were visualized by Oriole staining, cut from the gel and digested to enable tandem mass-spectrometry analysis.

Identification of tubule-associated host proteins

The proteomic analysis of the PM-tubule complex resulted in the identification of 143 proteins (Figure 3) of which 117 were host proteins, five viral proteins and 21 contaminants. As expected, CPMV MP and CPs were most abundantly present as judged from the iBAQ values. Relative protein abundance was quantified by calculating the difference between the mean LFQ-values of sample and control experiments. Lowered relative abundance of proteins was used as a measure of variability of protein abundance in our biological replicates (max log -1.9 or -82 fold), as reduced abundance is not expected from an enriched immunoprecipitation input. Therefore, proteins that had a logarithmic abundance ratio greater than +2, and were thus a 100 fold more abundant in the samples obtained with beads coated with MP-antibodies compaired to the pre-immune coated control, were considered candidate components of the PM-tubule complex.

Seven host proteins met all criteria mentioned (identified and encircled in Figure 3). These proteins were further characterized based on gene ontology annotation (Ashburner *et al.*, 2000) and sequence similarity (Table 2). These proteins belong to the families of heat shock proteins (HSP) 60 (I1LL24 and I1MJ28), ATPase-associated with diverse cellular activities (AAA-proteins) (I1LZ82), Δ1-pyrroline-5-carboxylate synthetase (P32296 and Q9AYM4), aminoacyl-tRNA synthetase (I1JMN8), and ribosomal proteins (I3T349). Molecular chaperone proteins HSP60 and AAA-proteins are encouraging discoveries, as these proteins are associated with membranes and have previously been linked to processes in viral infection.

Discussion

To identify host proteins that specifically associated with the CPMV movement tubule we performed a proteomic analysis of the PM-tubule complexes isolated from CPMV infected protoplasts. The annotation of identified peptides to proteins was done using a database composed of protein sequences from a higher taxonomical rank (order *Fabales*) which increased the size of our database considerably. The use of a larger database increases the chances to align identified peptides to proteins. However, the use of a wider range of plants for peptide annotation may lead to a bias towards conserved proteins, which in this analysis may be rational as host proteins required for tubule formation are thought to be conserved between taxonomical kingdoms (Kasteel *et al*., 1996).

All tubule associated proteins identified in this study are from conserved protein families. HSP60, or chaperonin, family members are molecular chaperones involved in a broad spectrum of cellular processes including protein folding, protein transport and protein complex assembly (Vierling, 1991; Hartl *et al*., 1992; Al-Whaibi, 2011). During virus infection chaperonins are involved in correct folding of viral proteins (Hildenbrand and Bernal, 2012; Snyder and Tarkowski, 2005) and were found to interact with polymerases of *Hepatitis B virus* (Park and Jung, 2001) and *Human immunodeficiency virus* (Bartz *et al*., 1994). Chaperonins specifically bind complexes of host and viral proteins formed during *Rice yellow mottle virus* infection (Brizard *et al*., 2006). Reports of chaperonins (HSP60) interacting with MPs are unknown, but a recent study on the spread of *Oilseed rape mosaic virus* in *Arabidopsis thaliana* showed that a mutation in a class II chaperonin, CCT8, reduced the spread of this *Tobamovirus* (Fichtenbauer *et al*., 2012). The authors hypothesise that CCT8 promotes viral cell-cell spread through correct folding of the transported MP complex. The role of chaperonin-assisted protein folding required for functionality of MP complexes could explain the presence of these proteins in our study.

The second type of conserved molecular chaperone identified in this study is an AAA-protein. These proteins exert mechanical force through ATP consumption, to fuel biological processes (Patel and Latterich, 1998; Santos, 2006). The AAA-protein I1LZ82 contains two copies of the archetypical AAA-domain and a two-part cell division cycle 48 (CDC48) domain. Studies in yeast and animals have

shown involvement of CDC48 proteins in cell division, protein translocation/recycling and membrane fusion (Meyer *et al.*, 2012; Yamanaka *et al.*, 2012). Little is known about plant CDC48 proteins, but they were shown to be involved in ER-membrane fusion (Latterich *et al.*, 1995) and the release of proteins from microtubules during cytokinesis (Cheeseman and Desai, 2004). Recently, Niehl *et al.* (2012) showed a direct interaction between a CDC48 protein and the MP of TMV. This arabidopsis CDC48b co-localizes with the MP at ER-inclusions and is required for extraction of the MP from this membrane, which results in MP localisation to the microtubule cytoskeleton (Niehl *et al.*, 2012). The CDC48 AAA-protein identified here could fulfil similar functions in the membrane-tubule complex, for example in translocation of MPs from the cytoskeleton or ER to the initiation sites of tubule assembly. The presence of the CDC48 protein in the PM-tubule complex could also be related to its function in alleviation of ER stress (Niehl *et al.*, 2012), which would occur upon removal of the desmotubule from the PD prior to tubule insertion. Alternatively, the presence of I1LZ82 could cohere with the assembly and maintenance of the tubule complex, analogous to the role of AAA-proteins in *Tomato bushy stunt virus* infection where, these proteins are required for the correct assembly of the replication complex (Barajas *et al.*, 2009).
The potential role of proteins P32296, Q9AYM4, I1JMN8, and I3T349 in the PM-tubule complex in plant virus movement, is less clear. It is conceivable that the presence of some of the proteins detected in our MS analysis could depend on an interaction with membrane fragments or membrane bound complexes residing in the PM-tubule complex, or even non-specific binding to the PM-tubule complex.
The Δ1-pyrroline-5-carboxylate synthase proteins (P32296, Q9AYM4) are part of the proline biosynthesis pathway and increased proline production confers osmotolerance in tobacco (Hu *et al.*, 1992; Kishor *et al.*, 1995). Whether these two proteins also protect plants against virus induced stresses is unknown, as is their mode of action.
The role of class-I aminoacyl-tRNA synthetase (I1JMN8) in viral infection is likely in the translation of proteins. tRNA synthetases are encoded by *Mimivirus* and packaged by some *Retroviruses* (Cen *et al.*, 2002; Abergel *et al.*, 2007). However, a role in intercellular transport has never been described for these synthetases.
The *Lotus japonicus* protein I3T349 is a structural component of the ribosome and although plant ribosomal proteins are generally upregulated upon infection (Yang

et al., 2009) its isolation with the PM-tubule structure is unexpected.
None of the host proteins known to interact with tubule-forming MPs were encountered in our analysis. The absence of proteins such as; MPI7, DNAJ-like protein and KNOLLE (Soellick *et al*., 2000; Huang *et al.*, 2001; Laporte *et al*., 2003) could be explained by the fact that these proteins do not localise to the PD or movement tubules. PDLPs (Amari *et al*., 2010; Thomas *et al*., 2008), however, are present in the PD and are essential for tubule formation by GFLV and *Cauliflower mosaic virus*. Their absence from our results could indicate that these proteins are not required for CPMV tubule formation or that the role of PDLPs is restricted to the PD and is dispensable in PD-less protoplasts. Fluorescent localisation studies involving PDLPs could confirm this hypothesis. (Chapter 5)
The seven host proteins that were identified in this study as part of the PM-tubule proteome do not include conventional trans-membrane proteins, which could imply that the proteins discovered here associate with the PM-tubule complex as part of a bigger complex. These proteins have at least a 100 fold greater abundance as compared to control experiments showing their strong affinity for the tubule complex. However, their significance in CPMV cell-cell transport needs further support from protein expression silencing experiments and *in vivo* localisation and interaction studies.

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Table 1: Host proteins known to interact with viral movement proteins, which were included in the custom protein sequence database used for peptide annotation.

Protein name	Uniprot code	Reference
PDLP 1-8	Q9ZU94, O22784, A8WAS5, Q6NM73, Q6NKQ9, Q0WPN8, Q8GXV7, Q6E263	Amari *et al.*, 2010 PLoS Pathog. 6:1-10
NtPME	Q9SC79	Chen *et al.*, 2000 EMBO J. 19:913-20
ATP8	Q8RY59	Lin and Heaton, 2001 J Gen Virol. 82:1245–1251
TIP1	Q9SAR5	Fridborg *et al.*, 2003 Mol Plant Microbe Interact. 16:132–140
Calreticulin	O04151	Chen *et al.*, 2005 Plant Physiol. 138:1866–1876
PAPK	Q3KT07	Lee *et al.*, 2005 Plant Cell 17:2817–2831
PAPK	Q8VYK9	Lee *et al.*, 2005 Plant Cell 17:2817–2831
SIUPTG1	Q6IV07	Selth *et al.*, 2006 Plant Mol Biol. 61:297–310
EB1a	Q7XJ60	Brandner *et al.*, 2008 Plant Physiol. 147:611 – 623
MPB2C	Q8S556	Kragler *et al.*, 2003 Virology 401:6 – 17
MPI7	P93829	Huang *et al.*, 2001 Plant Mol Biol. 47:663-675.
NbNACa1	A2PYH3	Kaido *et al.*, 2007 Mol Plant Microbe Interact 20:671 – 681
NtMPIP1	C4T7Z2	Shimizu *et al.*, 2009 Arch Virol. 154:959 – 967
NtCIP	Q6EIX9	Hofius *et al.*, 2007 J Virol. 81:11870 – 11880
AT4-1 (DNAJ-like)	Q1PE49	Von Bargen *et al.*, 2001 Plant Physiol Biochem. 39:1083 – 1093
cpHSC70-1	Q9STW6	Krenz *et al.*, 2010 Virology 401:6 – 17
NIG	Q8W4K6	Carvalho *et al.*, 2008 Plant J. 55:869 – 880
NtMBF1a	Q8S939	Matsushita *et al.*, 2002 J Exp Bot. 53:1531 – 1532
HFi22	Q8L8F9	Desvoyes *et al.*, 2002 Plant Physiol. 129:1521 – 1532

Table 1: Host proteins known to interact with viral movement proteins, which were included in the custom protein sequence database used for peptide annotation. (continued)

Protein name:	Uniprot code:	Reference:
fibrillarin 2	Q94AH9	Kim *et al.*, 2007 Proc Natl Acad Sci USA. 104:11115 – 11120
RIO kinase	Q75XU7	Yoshioka *et al.*, 2004 Mol Cells. 17:223 – 229
CK2	B2CY89	Modenat *et al.*, 2008 Virus Res. 137:16 – 23 Matsushita *et al.*, 2003 J Gen Virol. 84:497 – 505
LeNIK-like	Q9LFS4	Mariano *et al.*, 2004 Virology 318:24 – 31 Su *et al.*, 2003 Plant Cell Physiol. 41:1136 – 1142
2bip	Q9SPL9	Ham *et al.*, 1999 Mol Cells. 9:548 – 555
IP-L	Q9FXT0	Li *et al.*, 2005 Arch Virol. 150:1993 – 2008
PVIP	Q84N38	Dunoyer *et al.*,2004 J Virol. 78:2301 – 2309
eIF4Eiso	O04663	Léonard *et al.*, 2000 J Virol. 74:7730 – 7737 Léonard *et al.*, 2004 J Gen Virol. 85:1055 – 10632004
AtNSI	Q7X9V3	McGarry *et al.*, 2003 Plant Cell. 15:1605 – 1618 Carvalho *et al.*, 2006 Plant Physiol. 140:1317 – 1330
PSI-K	Q5EEQ1	Jiminez *et al.*, 2006 Mol PlantMicrobe Interact. 19:350 – 358
KELP	Q9AVE8	Matsushita *et al.*, 2001 MolCells. 12:57 – 66
NtCDC48	Q1G0Z1	Niehl *et al.*, 2012 Plant Physiol. 2012 160:2093-108
AtCDC48b	Q9ZPR1	Niehl *et al.*, 2012 Plant Physiol. 2012 160:2093-108
Synaptotagmin A	F4IFM6	Lewis and Lazarowski, 2010 Proc Natl Acad Sci USA. 107:2491 – 2496
RNB8	B0FFN7	Lu *et al.*, 2009 Virus Genes 2009 38:320-7
RNB5	Q84Q77	Lu *et al.*, 2009 Virus Genes 2009 38:320-7
Nt-Thaumatin-like	Q5DJS5	Kim *et al.*, 2005 Plant Mol Biol. 59:981-94
Nt-DNAJ-like	Q9SP09	Soellick *et al.*, 2005 Proc Natl Acad Sci USA. 97:2373-2378

Table 2: Host proteins associated with the tubular structure as identified by LC-MS/MS with Gene ontology proposed name and annotations. Ordered by logarithmic relative protein abundance.

Uniprot code	Log. rel. protein abundance	Proposed protein name	Organism	Gene ontology annotations			Blast prediction
				Biological process	Cellular component	Predicted molecular function(s)	
I1LL24	5.07	Uncharacterized protein	*Glycine max*	Protein refolding	Cytoplasm	ATP binding	HSP60 / chaperonin
I1LZ82	3.56	Uncharacterized protein	*Glycine max*	No prediction	No prediction	ATP binding	AAA-protein
I1MJ28	2.51	Uncharacterized protein	*Glycine max*	Protein refolding	Cytoplasm	ATP binding	HSP60 / chaperonin
P32296	2.45	Δ1-pyrroline -5-carboxylate synthase	*Vigna aconitifolia*	L-proline biosynthetic process	Cytoplasm	ATP binding, kinase activity, phosphate reductase activity	Δ1-pyrroline-5-carboxylate synthase
I1JMN8	2.41	Uncharacterized protein	*Glycine max*	Tryptophanyl-tRNA aminoacylation	No prediction	ATP binding, tRNA-acylation	Class-I aminoacyl-tRNA synthetase family
Q9AYM4	2.35	Δ1-pyrroline -5-carboxylate synthase	*Vigna unguiculata*	Proline biosynthetic process	Cytoplasm	ATP binding, kinase activity, phosphate reductase activity	Δ1-pyrroline -5-carboxylate synthase
I3T349	2.04	Uncharacterized protein	*Lotus japonicus*	Translation	Ribosome	Structural constituent of ribosome	50S ribosomal protein L19

Chapter four

Silencing of two host chaperone proteins negatively affects *Cowpea mosaic virus* infection dynamics.

Abstract

Proteomic analysis of movement tubule complexes isolated from *Cowpea mosaic virus* (CPMV) infected protoplasts showed that several host proteins co-purified with these viral structures. Among these were host proteins that belong to families of conserved chaperone proteins: the heat shock proteins (HSP)60, the 'ATPases Associated with diverse cellular Activities' proteins (AAA-proteins) and an HSP70. In this study the possible regulatory role of these household proteins in CPMV infection is investigated via a gene silencing approach through the synthesis of antisense RNAs. In *Nicotiana benthamiana,* expression of these proteins was silenced by the use of a virus-induced gene silencing system. Knockdown of both the HSP70 and AAA-protein expression resulted in a significant reduction of CPMV titres in infected leaves. Additionally, the silencing of AAA-protein and HSP70 also resulted in reduced spread of CPMV and TMV in infected leaves. These effects were not observed when silencing HSP60, which suggests a biologically relevant role for HSP70 and AAA-proteins in virus infection dynamics.

Introduction

Viruses require an intimate molecular interaction with their host in order to replicate and spread. In recent years a multitude of interactions have been identified which are thought to be required for intercellular transport of viruses in plants (for an overview see Niehl and Heinlein 2011; Harries and Ding, 2011). *Cowpea mosaic virus* (CPMV) is a well-studied plant virus, but host components that interact with CPMV during infection or transport are currently unknown. In a proteomic analysis of CPMV's intercellular movement complexes, that consist of virally encoded virion-containing tubules surrounded by plasma membrane, which were formed on the surface of infected protoplasts, 117 host proteins were identified (Chapter 3). Seven of these proteins were shown to be present in high abundance, at least a 100 fold more than in the control, and were considered to be specifically associated with the movement complex.

To determine whether the presence of these host proteins has a biological significance in the intercellular movement of CPMV, a selection of the three most abundant complex-associated proteins was further analysed. Two of these belong to the heat shock protein (HSP)60 (or chaperonin) family, and the third protein belongs to a family of 'ATPases Associated with diverse cellular Activities' proteins (AAA-protein). In addition, although it did not meet the abundance threshold, a HSP70 was selected. This protein was selected because its abundance and HSP70s have been shown to be required for both viral infection (Wang *et al.*, 2009; Mine *et al.*, 2012) and intercellular transport (Alzhanova *et al.*, 2001; Krenz *et al.*, 2010).

Homologs of HSP60 and AAA-proteins have diverse biological functions and have previously been associated with viral infection and transport. HSP60 or chaperonin proteins are molecular chaperones involved in a broad spectrum of cellular processes including protein folding, protein translocation, the assembly of protein complexes, and modulation of receptor activities (Vierling, 1991; Hartl *et al.*, 1992; Al-Whaibi, 2011). These proteins are known to interact with polymerases of *Hepatitis B virus* (Park and Jung, 2001) and *Human immunodeficiency virus* (Bartz *et al.*, 1994) and specifically bind cytoplasmic replication complexes of *Rice yellow mottle virus* (Brizard *et al.*, 2006). Chaperonin proteins facilitate correct folding of viral proteins (Snyder and Tarkowski, 2005;

Hildenbrand and Bernal, 2012) and mutations to a type II chaperonin reduced *Tobamovirus* spread in *Arabidopsis thaliana* (Fichtenbauer *et al.*, 2012).

AAA-proteins are molecular chaperones that exert mechanical force through ATP consumption in order to fuel biological processes, such as protein complex formation and protein transport across membranes (Patel and Latterich, 1998; Santos, 2006). The identified AAA-protein belongs to the cell division cycle protein 48 (CDC48) subfamily which, in yeast and animals, has been shown to be involved in cell division, protein translocation, protein recycling and membrane fusion (Meyer *et al.*, 2012; Yamanaka *et al.*, 2012). In plants CDC48 proteins were shown to function in ER-membrane fusion (Latterich *et al.*, 1995) and cytokinesis (Cheeseman and Desai, 2004). In plant virus infection, AAA-proteins were found to aid virus accumulation (Abbink *et al.*, 2002) and support the assembly of replication complexes (Barajas *et al., 2009)*. Furthermore, Niehl *et al.* (2012) showed that CDC48 proteins are involved in translocation of movement protein complexes and are thought to relieve virus induced ER stress.

The third molecular chaperone selected for further analysis was a HSP70 protein. Like the other molecular chaperones, HSP70 proteins have a broad spectrum of cellular functions and are well known for their protection from heat stress and their function in protein folding (Rajan and d'Silva, 2009; Mayer, 2013; Saibil, 2013). These proteins are also involved in translocation of cargo across membranes (Soll and Schleiff, 2004) and interact with viral proteins (Mathioudakis *et al.*, 2012; Gorovits *et al.,* 2013). In plant virus infection HSP70 proteins were shown to be involved in the replication of *Red clover mosaic virus* (Mine *et al.*,2012), *Potato virus A* (Hafrén *et al.*, 2010) and *Tomato bushy stunt virus* (Wang *et al.*, 2009), with functions in correct replicase-complex folding, regulation of replication and membrane insertion of the replication complex respectively. In *Closterovirus* intercellular transport, a virally encoded HSP70 homologue localizes to the PD and was shown to be essential for virus transport (Alzhanova *et al.*, 2001). In *Abutilon mosaic virus* infection a direct interaction of the movement protein (MP) with a HSP70 was shown *in planta* at the periphery of cells (Krenz *et al.,* 2010), indicating a functional relevance of this chaperone in cell-cell movement. The fact that HSP70s have motor activities that drive protein translocation (Pilon and Schekman, 1999; Voisine *et al.*, 1999) suggest that HSP70 proteins may have the ability to chaperone viral protein (complexes) to

neighbouring cells through plasmodesmata (Boevink and Oparka, 2005).
Considering these three chaperone proteins co-precipitated with the CPMV movement complex and have diverse cellular functions in protein (complex) formation, translocation and membrane insertion, together with their associations to viral infection and movement, it is expected that these host proteins could have biological significance in the infection of CPMV *in planta*. To test this hypothesis, expression of close homologs to these HSP60/70 and CDC48 AAA- proteins were silenced in *N. benthamiana* plants and the effect of this silencing on virus accumulation and spread in leaves was analysed.

Materials and methods

Assembly of virus induced gene silencing constructs

The nucleotide (nt) sequence belonging to proteins identified in a previous proteomic analysis of the membrane-tubule complex (Chapter 3) served as a template for primer design to amplify homologous gene fragments in *N. benthamiana* (Nb) (Table 1). These primers amplified products from *N. benthamiana* cDNA which was generated from total RNA using a poly-A-tail binding primer and *Avian Myeloblastosis Virus* reverse transcriptase (Promega) according to the manufacturers instructions. Sequence annotation of amplified products by BLAST searches against the non-redundant nucleotide database (NCBI), revealed that multiple fragments could be amplified for HSP60 and HSP70 and showed that single fragments were amplified for CDC48 and phytoene desaturase (Table 1). A GFP fragment was amplified from plasmid DNA (pMON-MP-GFP, Pouwels *et al.*, 2002).
The amplified 303-608nt PCR silencing-fragments were either ligated into a pJET 1.2 vector (Thermo Scientific) or used directly in a Gateway® (Invitrogen) BP-reaction with the pDONR207 entry vector (Figure 1A). After amplification and selection of the obtained pDONR207 plasmids, these were recombined into the GATEWAY®-compatible TRV2 destination vector described by Liu *et al.* (2002). The recombined TRV2 plasmids (pTRV2) were co-transformed with TRV1 plasmids (pTRV1) into the hyper virulent *Agrobacterium tumefaciens* strain COR-308 (Hamilton *et al.*, 1996) to induce transient silencing of gene expression by *Agrobacterium tumefaciens* infiltration (de Ronde *et al.*, 2013 (Figure 1)). Control

plants were infiltrated with pTRV1 and a pTRV2 carrying either a phytoene desaturase (PDS) or GFP silencing-fragment, or infiltrated with buffer.

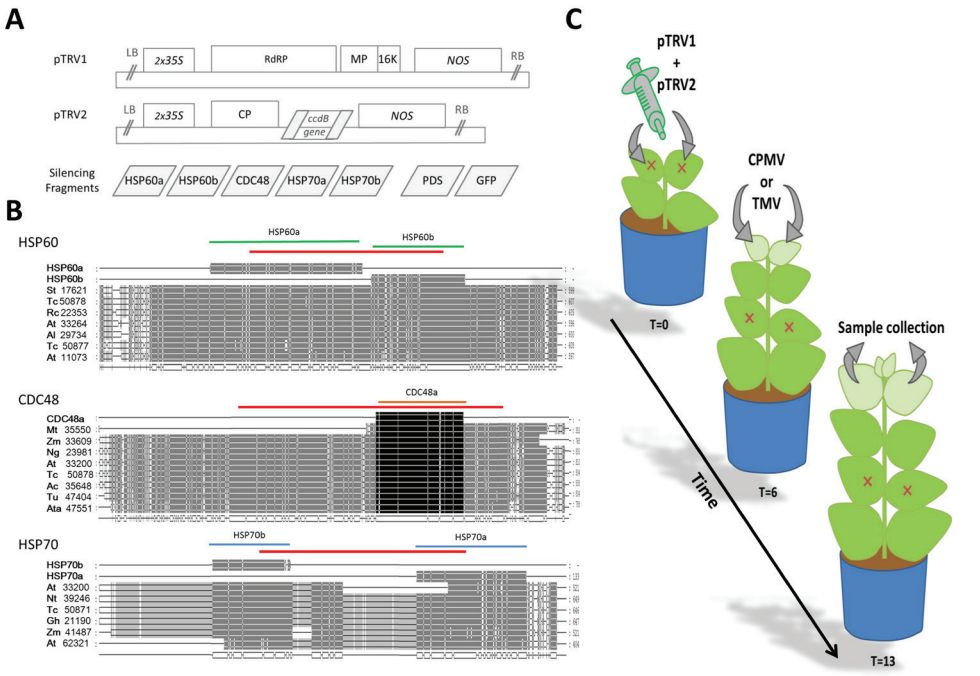

Figure 1: Set-up of the virus induced gene silencing experiment.
A) Schematic representation of pTVR1 and pTRV2. The constitutive promoter 35S (from *Cauliflower mosaic virus*) is combined with the nopaline synthase gene terminator (NOS). pTRV1 encodes; RdRP (RNA dependent RNA polymerase), MP and 16K protein, pTRV2 encodes the CP (coat protein) and the gateway adaptors sequences (indicated in grey) flanking the ccdB-gene which is replaced by the PCR amplified silencing fragments. LB= left border, RB= right border (adapted from Liu *et al.*, 2002). **B)** Alignment of silencing fragments to HSP60, HSP70 and CDC48 proteins from various organisms, species abbreviations: St (*Solanum tuberosum*), Tc (*Theobroma cacao*), Rc (*Ricinus communis*), At (*Arabidopsis thaliana*), Al (*Arabidopsis lyrata*), Mt (*Medicago truncatula*), Zm (*Zea mays*), Ng(*Nicotiana glutinosa*), Ac (*Allium cepa*), Tu (*Triticum urartu*), Ata (*Aegilops tauschii*), Nt (*Nicotiana tabacum*), Gh (*Gossypium hirsutum*), numbers following species abbreviations are part of the protein accession numbers. Alignment of the HSP60 and HSP70 a and b fragments shows that both fragments are found in the same protein sequence. Green, orange and blue lines represent PCR amplified silencing fragments, red lines indicated fragments amplified for mRNA detection. **C)** Schematic representation of *N. benthamiana* silencing. Red crosses indicate the site of *A. tumefaciens* (with pTRV 1+2) infiltration, silencing symptoms in systemic leaves at 6 and 13 days post ATTA are represented by pale green leaf colour.

Virus induced gene silencing (VIGS)

N. benthamiana plants were grown on soil under greenhouse conditions at 23°C (± 2°C) with 8 h of (day)light. Four week-old plants were subjected to agrobacterium transient transformation assays (ATTA, de Ronde *et al.*, 2013). A 1:1 mixture of pTRV1 and pTRV2 containing *A. tumefaciens* bacteria (at an OD_{600} of 0.5) was pressure infiltrated in the apoplast of the third and fourth true leaf of the plants to induce silencing. Six days post infiltration leaf seven and eight were mechanically inoculated with 0.5 µg CPMV.

Semi-quantitative RT-PCR

To assess mRNA levels in the systemically silenced leaves, total RNA was extracted from 0.1 g of *N. benthamiana* leaf material at 10 d post ATTA using Trizol (Invitrogen). RNA quality and quantity was checked by 1% agarose gel electrophoresis and by photospectrometry (Nanodrop, Thermo scientific) respectively. cDNA was generated with 2 µg of RNA input using *Avian Myeloblastosis Virus* reverse-transcriptase (Promega) as instructed by the manufacturer. Two µl of 20µl total cDNA synthesis reaction volume was used in a PCR with fragment-connecting primers for HSP60a,b and HSP70a,b detection and fragment-flanking primers for CDC48, GFP and PDS detection (Table 1). After 32 cycles of PCR amplification relative intensities of PCR products were calculated using Image lab 5.0 (Bio Rad Laboratories). Variation in template amounts were corrected for using amplified actin cDNA intensities as loading control.

Measurement of virus spread in systemically silenced leaves

To assess viral spread, a transgenic fluorescent clone of CPMV (M19GFP2A, described by Gopinath *et al.*, 2000) or TMV (pBin TMV-GFP-30B, described by Lacorte *et al.*, 2010) was used, henceforth referred to as CPMV-GFP and TMV-GFP respectively. Systemically silenced leaves were mechanically inoculated with 0.5 µg CPMV-GFP, or 50 µl of homogenised TMV-GFP infected leaf material. Infection foci sizes were measured at 4 d post infection (dpi) using a stereo microscope (Leica MZFL3) suited for GFP imaging, equipped with a TCC-1.4ChICE camera (Tucsen Imaging, China). Spot size was determined by manual tracing of the fluorescent spot contour using the polygon selection tool of ImageJ (version 1.48d, Schneider *et al.*, 2012).

Table 1: Overview of primers used for amplification of silencing target sequences from *N. benthamiana* and mRNA detection.

	Uniprot code	Primers used (5′ to 3′) Forward (Fw) and Reverse (Rv)	Product size	Amplified product BLAST prediction	*Sequence conservation (nt,AA)**	pTRV2 clone name
Silencing fragment amplification	B1NYI4	Fw:gcaatggaaggaacattc Rv:agtctctcaggaggattacc	420 nt	*Nicotiana benthamiana*, phytoene desaturase	*100%,100%*	TRV-PDS
	P42212	Fw:gaagaacttttcactggagttgtc Rv:gttcccgtcgtccttgaa	303 nt	*Aequorea Victoria*, green-fluorescent protein	*100%,100%*	TRV-GFP
	I1LL24, I1MJ28	Fw:gctaccaatgatgtggctggt Rv:tcaccaaaaccaggggct	608 nt	*Solanum tuberosum*, chaperonin-60 beta	*92%,90%*	TRV-HSP60a
	I1LL24, I1MJ28	Fw:caacccttactggaggcact Rv:caccctcctccaacaacaat	373 nt	*Solanum tuberosum*, chaperonin-60 beta	92%,91%	TRV-HSP60a
	I1JXA0	Fw:tgatattggaggtcttgagaatg Rv:gataatgtcaggtctgttggttg	457 nt	*Nicotiana tabacum*, putative spindle disassembly related protein CDC48	*91%,98%*	TRV-CDC48
	G7KZM0	Fw:ggtgaagattttgataacagaatgg Rv:accaacaagaacaacatcatgaac	332 nt	*Solanum lycopersicum*, heat shock protein cognate 70	*93%,92%*	TRV-HSP70a
	G7KZM0	Fw:attgatgccaatggtatcttga Rv:ccttggtacatctttgcaat	401 nt	*Nicotiana tabacum*, Heat shock protein 70	*98%,92%*	TRV-HSP70b

	Detection of:	Primers used (5′ to 3′)	Product size
mRNA detection	Nb-HSP60	Fw:ACTGGTTGACAAGAAGATAACAAATG Rv:CAACAGCTGCCTTTGTTGC	684 nt
	Nb-CDC48	Fw:GGCAAAGACACACATGGCTA Rv:GATCTCAGGCACCTCATCGT	988 nt
	Nb-HSP70	Fw:GATGTTGTTCTTGTCGGTGG Rv:GTCCAGTGGTCTTGTCCTCG	483 nt
	Nb-PDS	Fw:CTCACGCCCAACTAAACCAT Rv:TCTTTCCAGTCTTCAGGCAAA	827 nt

*Coverage of fragments is ≥99%

Measurement of virus accumulation in systemically silenced leaves

To determine the viral titre, CPMV-inoculated leaves were harvested at seven dpi, leaf halves were ground and diluted 30 times (weight/volume) in PBS before loading in a coated 96 wells ELISA plate (Greiner Bio-One). Double antibody sandwich ELISA tests were adapted from Clark and Adams (1977) as follows: plates were coated with CPMV-IgG (1 µg/ml) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3, pH 9.6) overnight at 4°C. Plates were rinsed three times with demineralized water. Samples were loaded at least in duplo, along with a mock infected leaf homogenate as negative control and purified CPMV (0.15 mg/ml) as positive control. Plates were incubated at 37°C for 2 h. After rinsing, alkaline phosphatase conjugated anti-CPMV antibody was added and incubated at 37°C for 2 h. Plates were washed three times and 1 µg/ml para-nitrophenylphosphate in substrate buffer (9.6% diethanolamine, 3 mM NaN_3, in H_2O, pH 9.8) was added. Plates were kept at room temperature for 60 min before absorbance was measured at 405 nm using a FLUOstar OPTIMA (BGM-Labtech).

Statistical analysis

Statistical tests were performed using IMB SPSS statistics software package version 21. Significant difference between the measured infection foci size in the different genetic backgrounds were tested as follows: data was checked for normality and equality of variance using a Kolmagorov-Smirnov and Levene's tests respectively. Square root transformed CPMV-GFP foci size data were normally distributed but equal variance could not be assumed, therefore a non-parametric Dunnett's T3 post-hoc pair-wise comparison was used to determine the significance of differences between treatments. TRV-GFP foci size measurements were non-normally distributed and transformation of the data did not change this. Significant differences among treatments was tested by comparing infection foci size of the TRV-PDS control with the other treatments by individual non-parametric Mann-Whiney-U tests. ELISA values were normally distributed and of equal variance, significance was therefore tested using a non-parametric Kruskal–Wallis test.

Results

Silencing of host chaperone genes causes altered leaf morphology

To investigate whether the HSP60, HSP70 and AAA-protein chaperones identified in the MS/MS analysis are of biological significance in CPMV infection and/or intercellular movement, we knocked down their expression by VIGS (Baulcombe, 1999; Ratcliff *et al.*, 2001). To target the identified proteins by VIGS, homologous gene fragments were picked up from *N. benthamiana* transcripts and inserted in the TRV amplicon system (Figure 1A; Liu *et al.*, 2002). We used *N. benthamiana* for our studies as this host supports both TRV and CPMV systemic infection. Comparative analysis of the sequences inserted into TRV2 plasmid (pTRV2) against the non-redundant nucleotide database using the BLASTn algorithm (NCBI) showed that the amplified fragments coded for HSP60 (two fragments), HSP70 (two fragments) and a CDC48 type AAA-protein. The translated HSP60 and HSP70 fragments could be aligned to single HSP sequences (using BLASTx algorithm, NCBI) indicating these fragments are likely to target the same host protein transcript (Figure 1B, Table 1).

TRV2-vectors (pTRV2) carrying fragments of HSP60 (TRV-HSP60a/b), HSP70 (TRV-HSP70a/b) and CDC48 protein genes (TRV-CDC48), were introduced in young *N. benthamiana* plants by *Agrobacterium tumefaciens* transformation. The onset of systemic silencing was tested using a fragment of the phytoene desaturase (PDS) gene in the TRV backbone (TRV-PDS). The PDS gene functions in carotenoid biosynthesis and its silencing leads to chlorosis/bleaching, a visible confirmation of effective gene silencing (Kumagia *et al.*, 1995). ATTA of the third and fourth true leaf resulted in visible PDS silencing in leaf seven and eight at seven days post infection (dpi) (Figure 1C). This indicated that silencing was effective and is established in the third and fourth leaf above those infiltrated.

Ten days post ATTA endogenous gene silencing resulted in phenotypic changes in most leaves (Figure 2A). Stunted growth was observed in all TRV infected plants. Silencing of the three endogenous chaperones resulted in additional phenotypic changes in the host. In HSP70 and CDC48 silenced plants, newly formed leaves were underdeveloped and prolonged silencing (16-18 days) inhibited the formation of new leaves through loss of the shoot apical meristem, a phenomenon previously described for HSP70 silenced *N. benthamiana* plants

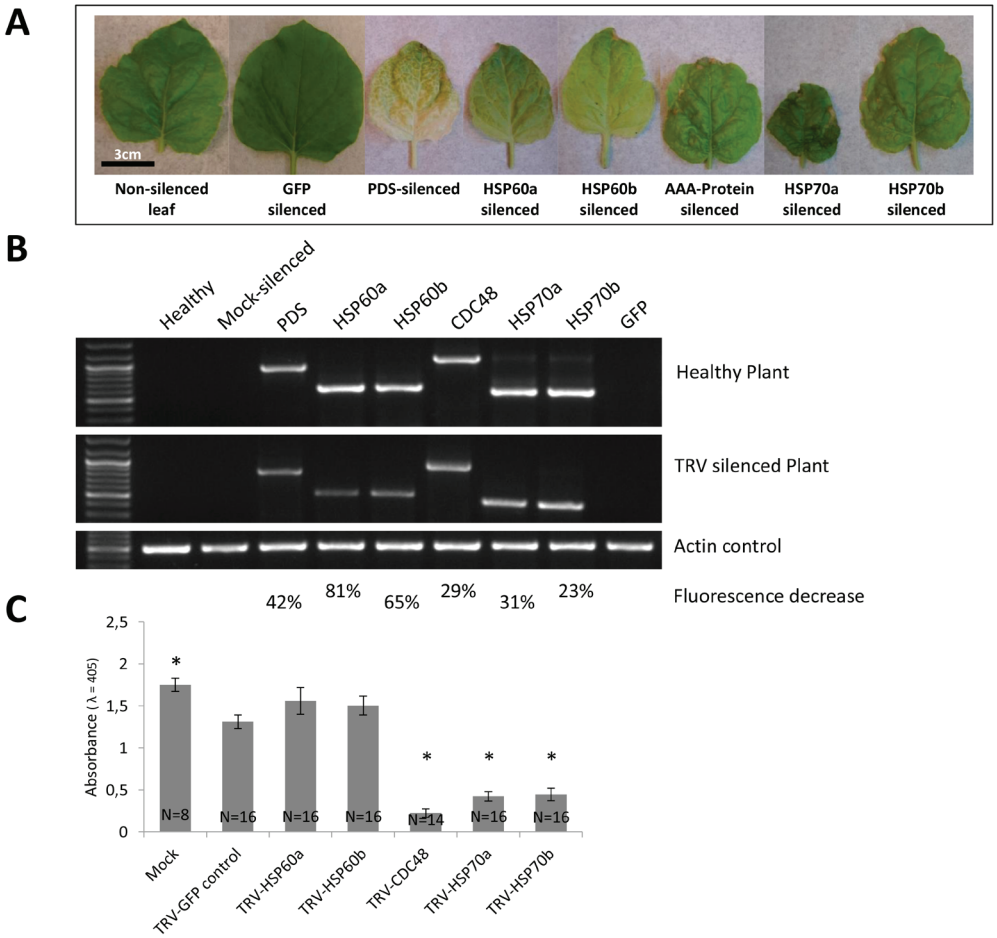

Figure 2: TRV induced gene silencing symptoms in N. benthamiana plants.
A) Phenotypes observed at 10 days post ATTA in leaves that were systemically silenced with different VIGS constructs. **B)** Semi-quantitative RT-PCR showing mRNA transcript levels of genes targeted by the indicated VIGS constructs. PCR primers for amplification of GFP were combined with healthy, mock silenced and GFP silenced plant cDNA, absence of bands confirms the absence of this gene from the genome, and shows that the possible presence of viral RNA does not interfere with detection (no band in GFP-TRV sample). Actin amplification was used as an input benchmark to correct for possible variation in cDNA input, repersentative blot is shown, percentages are averaged values from 3 seperate experiments. **C)** Quantification of CPMV titre in systemically silenced leaves at seven dpi by ELISA. The results of three repeated individual experiments were pooled. * Indicates a significant difference in virus titre in comparison to the TRV-GFP control.

(Chen *et al*., 2008). Silencing of CDC48 resulted in brittle leaves, an effect which has been previously described for CDC48 silencing (Bae *et al.*, 2009). Silencing of HSP60 did not show adverse effects on plant development, these plants developed like the TRV infected control plants, with a mild chlorosis observed in silenced leaves.

Phenotypic effects of silencing were biochemically confirmed by semi-quantitative RT-PCR. This analysis showed a 23-81% decrease of the presence of the targeted mRNA in silenced leaves at 10 days post ATTA (Figure 2B). PDS transcripts were reduced by 42%, which is in accordance with the symptoms observed on leaves as these were not fully bleached at the time of sampling (Figure 2B and 2A). Levels of gene silencing were not very strong for CDC48 and HSP70, which might correspond to a lower degree of nucleotide sequence conservation in the targeted regions or might be caused by redundancy in gene product function, as in *Arabidopsis thaliana* for example eight HSP70 genes are predicted from the genome sequence (Al-Whaibi, 2011).

Knock down of HSP70 and CDC48-proteins alters CPMV infection dynamics

To find whether the knockdown of chaperone proteins has an effect on the accumulation of CPMV, systemically silenced leaves were infected with CPMV. Seven days after infection these leaves were harvested and viral titres were determined from equal amounts of material using ELISA. Measurements from repeated experiments showed diverging titres of CPMV when targeting different genes by VIGS (Figure 2C). To interpret the impact of gene silencing on CPMV replication several control experiments were performed. CPMV infection of mock silenced plants allows comparison of virus accumulation in silenced and non-silenced plants. To correct for the possible effect of the presence of both *A. tumefaciens* and TRV, TRV vectors carrying the non-endogenous GFP protein were used in ATTAs. An empty pTRV2 vector was not used as control as this vector was shown to display altered infection kinetics, in comparison to gene targeting pTRV2 vectors, due to their smaller size (Wu *et al.*, 2011).

A clear decrease in viral titre was found when either CDC48 or HSP70 genes were targeted for silencing (Figure 2C), even though the levels of gene silencing were quantified at only 23-31% below the control. Silencing of HSP70 by either of the two TRV-clones resulted in a threefold reduction in viral titre and knock-down of

the CDC48 AAA-proteins resulted in an even stronger, nearly six fold, reduction of viral titre in comparison to the TRV-GFP control. These significant decreases in virus titre suggests that lowered abundance of both CDC48 and HSP70 reduces viral accumulation. Silencing of HSP60 genes did not decrease virus accumulation in these leaves. Viral titres were even slightly higher in silenced leaves, however, this increase was not significant.

Because the targeted host proteins were identified through their co-precipitation with the CPMV movement complex (Chapter 3), the observed effect of HSP70 and CDC48 silencing could be a result of reduced viral spread. To further investigate this possibility, intercellular spread of a GFP encoding CPMV strain was monitored in the different silenced backgrounds. Fluorescent infection foci could be detected from three dpi onwards and were quantified at four dpi (Figure 3). Because GFP expression was monitored, TRV-GFP could not be used as a control for the possible negative effect of bacteria and TRV in these experiments. Therefore, the TRV-PDS clone was used as a control in these experiments, assuming that silencing of PDS does not influence viral infection. Silencing with either of the HSP60 targeting constructs caused only a marginal and non-significant reduction in infection foci size as compared to the foci formed in control plants (Figure 3B), which suggests that HSP60 proteins are non-essential for CPMV spread. Silencing of CDC48, however, did have a significant effect on infection foci size, the CPMV-GFP infections were restricted to a smaller area with an average reduction in size of 2.5-fold. Silencing of HSP70 also resulted in a significant reduction of infection foci size. When compared to the TRV-PDS control plants, silencing with either of the HSP70 (a/b) constructs led to an approximate 1.6 fold reduction in infection foci size (Figure 3B). The reduced size of infection foci upon HSP70 and CDC48 silencing is in line with the observed decrease in virus titre. Silencing of HSP70 and CDC48 thus causes a significant reduction in virus titre and spread, in which CDC48 silencing shows the strongest effect.

TMV spread is also affected by silencing of CDC48 and HSP70 chaperones

The observation that silencing of either CDC48 or HSP70 affects both accumulation and cell-cell spread of CPMV raised the question whether these effects were specific to viruses that transport their virions between cells by a tubule-mediated mechanism. To address this question we investigated the spread of a fluorescent strain of TMV in these silenced backgrounds. Unlike CPMV, TMV does not make use of tubular structures to transport its genome to neighbouring cells, but instead shuttles viral-ribonucleoprotein complexes through dilated plasmodesmata (Liu and Nelson, 2013). When silenced *N. benthamiana* leaves were infected with TMV-GFP a diverse range of infection foci sizes was found in the different treatments (Figure 3C). Interestingly, the general trend of the treatments was similar to that observed for CPMV infection (compare treatments in Figure 3B and Figure 3C). In comparison to the TRV-PDS control, no significant difference in infection foci size was found for the HSP60 silenced plants. However, a significant reduction in infection foci size was found

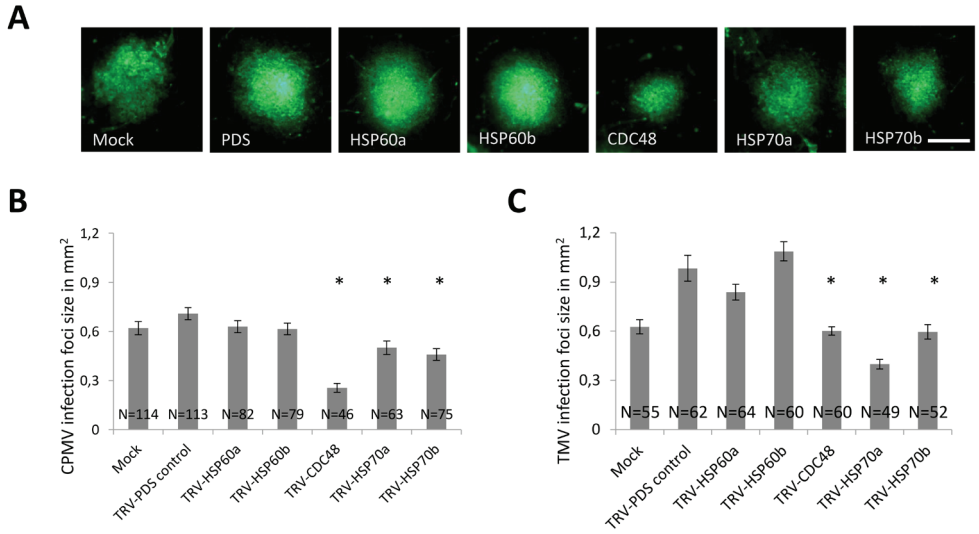

Figure 3: Effects of gene silencing on CPMV infection dynamics.
A) Representative CPMV-GFP infection foci, portraying size and intensity of foci in various silenced backgrounds. **B)** Quantification of the size of CPMV-GFP infection foci. **C)** Quantification of TMV-GFP infection foci size. Data in B and C represent averages of at least three individual experiments. Error bars represent standard error. * Indicates significant difference in foci size of treatments in comparison to the TRV-PDS control. Scale bar = 0.5 mm

in both HSP70 and CDC48 silenced backgrounds, as was observed in CPMV-GFP infection (Figure 3C). Thus, our experiments show that silencing of either CDC48 or HSP70 results in significant reduction of infection foci size for both tubule-forming (CPMV) and non-tubule forming viruses (TMV).

Discussion

We have investigated the potential role of host proteins from three families of molecular chaperones, i.e. HSP60, HSP70 and CDC48 AAA-proteins, in CPMV infection of *N. benthamiana*. These host proteins were found to be abundantly present in isolated viral movement complexes (Chapter 3), suggesting a possible role for these proteins in CPMV tubule-guided movement. Down-regulation of the expression of HSP70 and the AAA-protein CDC48 resulted in reduced virus accumulation in *N. benthamiana* leaves as well as a reduction of the size of infection foci. These findings suggest a correlation between the expression of HSP70 and CDC48 proteins and the accumulation and spread of CPMV in *N. benthamiana*. Hampered spread in an HSP70 or CDC48 silenced background was also observed for TMV. These results suggest that viruses that move between cells by means of movement tubules (CPMV) ánd viruses that transverse the PD as viral-ribonucleoprotein complexes (TMV) benefit from the presence of HSP70 and CDC48 proteins.

The proteins identified in the proteomic analysis of the CPMV movement complex originated from cowpea (*Vigna unguiculata*) protoplasts (Chapter 3). Gene fragments required for VIGS were obtained from *N. benthamiana* and cloned in the TRV VIGS system. The amplified gene fragments showed high levels of conservation to the targeted gene sequences, both on nucleotide (≥91%) and on protein (≥90%) level (Table 1). This high degree of sequence conservation suggests that the genes targeted in the *N. benthamiana* background are indeed homologues to the cowpea sequences identified initially.

Comparative analysis of mRNA levels in silenced leaves versus healthy leaves by semi-quantitative PCR showed a reduction of target gene mRNA levels of 23% to 81% percent (Figure 2B). High levels of silencing correspond to strict nucleotide conservation in the targeted region. This is likely the case for HPS60. The limited decrease in mRNA levels upon silencing of HSP70 or CDC48 could either be due

to a lower degree of nucleotide conservation in the targeted regions or mRNA decrease could be masked by amplification of paralogous genes. However, the observed effects of HSP70 and CDC48 silencing on virus dynamics are clear and significant. Although we cannot exclude that the detrimental effects observed after prolonged host gene silencing had an effect on earlier stages of viral infection, no relation was found between the severity of phenotypic effects and reduction of viral titre or viral spread.

At this time it is unclear whether the effects observed upon silencing of HSP70 and CDC48 influences virus intercellular spread directly, or whether the reduced virus spread is an indirect consequence of reduced viral replication, leading to delayed virus spread. We have attempted to differentiate between the effects of silencing on intercellular-transport and replication by different experimental approaches. By infecting protoplasts generated from HSP70 and CDC48 silenced *N. benthamiana* leaves, levels of tubule formation and virus replication in these protoplasts can be compared to the corresponding levels of replication and tubule formation in non-silenced protoplast. However, the silenced protoplasts did not survive CPMV infection and we could therefore not differentiate between effects of silencing on either replication or tubule formation. Experiments to uncouple tubule formation and replication in protoplasts by chemical inhibition of HSP70 (by addition of Quercetin as previously done by Wang *et al.* 2009 and Hafrén *et al.* 2010), did not provide conclusive data. Detection of HSP70 and CDC48 proteins by immuno-gold labelling of isolated tubules, did not show any specific labelling, possibly because the heterologous anti-HSP70 and anti-CDC48 antibodies do not recognise the respective *N. benthamiana* protein isoforms.

The effects of gene silencing on virus spread are illustrated by the sizes of fluorescent infection foci shown in Figure 3. Silencing of HSP60 did not significantly affect CPMV infection dynamics, nor did it affect the spread of TMV-GFP (Figure 3C). Silencing of a cytoplasmic chaperonin (CCT8) has recently been reported to hamper *Tobamovirus* spread (Fichtenbauer *et al.*, 2012). The role of CCT8 in intercellular transport is thought to relate to (un)folding of complexes to enable their transport across plasmodesmata (Fichtenbauer *et al.*, 2012). Assistance in the correct formation of multi-protein complexes would explain the presence of this HSP60 at the interface of the movement tubule and the plasma membrane during CPMV infection. The absence of observed effects on virus

replication or spread upon silencing of our HSP60 homologues could indicate that the HSP60 targeted in this study is not a cytoplasmic (type II) chaperonin protein, but may instead belong to the type I chaperonins that are mainly found in chloroplasts (Ranson *et al.*, 1998), which could indicate the interaction of these proteins with the membrane-tubule complex was non-specific.

Silencing of the AAA-protein CDC48 causes a reduction in CPMV accumulation. This is in accordance with earlier described results where silencing of AAA-proteins resulted in lower titres of *Alfalfa mosaic virus*, *Potato virus X* and TMV (Abbink *et al.*, 2002). In our experiments we also observed that reducing CDC48 transcript levels resulted in smaller infection foci at 4 dpi, which could suggests that intercellular spread is hampered as a result of silencing. Recently, Niehl *et al.* (2012) showed an interaction between an arabidopsis CDC48 and the MP of TMV at ER-microtubule intersections. This interaction was thought to be required for the regulation of TMV intracellular transport by establishing an equilibrium that regulates the switch between TMV accumulation and intercellular movement (Niehl *et al.*, 2013). Interestingly, in the study by Niehl *et al.* (2012) the overexpression of arabidopsis CDC48B resulted in reduced virus spread, whereas in our experiments downregulation of native CDC48 expression resulted in a reduction of virus spread. The different results in our study and the study of Niehl *et al.* (2012) could be due to the differences between intercellular spread of CPMV and TMV. Given that CDC48 interacts with the TMV MP at ER-microtubule junctions and both ER and microtubules seem dispensable for tubule formation by the CMPV MP (Pouwels *et al.*, 2002), the interaction of CDC48 with the MP could serve distinct functions in infection of these two viruses. The contradicting effects of CDC48 expression levels on the spread of TMV in our study and that of Niehl *et al.* (2012) are surprising. It is possible that the different methods of manipulating transcript levels, over-expression of a specific gene by Niehl *et al.* (2012) and silencing of all transcripts with a homologous sequence in this study, do not produce the expected opposing outcome due to the fine-tuned regulatory role of CDC48 proteins in TMV spread and accumulation. Experimental data on the localisation of CDC48 isoforms and their interaction with CPMV MP are required to determine the role of this intriguing host protein in CPMV infection and intercellular transport. Establishing the site of the CDC48-MP interaction is also required to discern whether CDC48 silencing affects CPMV replication

or intercellular-transport. Because AAA-type ATP-ases have been shown to be required in formation of *Tomato bushy stunt virus* replication complexes (Barajas *et al.*, 2009) the former function cannot be ruled out.

Hsp70 isoforms are thought to be involved in replication (Wang *et al.*, 2009; Nagy *et al.*,2011) and cell-cell transport of plant viruses (Alzhanova *et al.*, 2001; Krenz *et al.* 2010). Silencing of HSP70 is known to negatively affect replication of plant viruses from the genus *Tombusvirus* (Serva and Nagy, 2006) and *Tobamovirus* (Chen e*t al.*, 2008), here we have shown that also the accumulation and spread of CPMV is hampered by silencing of HSP70 isoforms. The molecular mechanisms underlying the observed effects of HSP70 silencing are unknown at this time. As suggested for the CDC48 proteins further experimental studies should focus on (co-)localisation of MPs and the HSP70 in healthy and CPMV-infected cells. Techniques such as bi-molecular fluorescent complementation (BiFC) or Förster resonance energy transfer (FRET) could be used to test both the site of co-localisation and the (possible) interaction between these proteins.

In conclusion, in this paper we report a relevance of host (chaperone) proteins in CPMV infection. Silencing the expression of HSP70 and CDC48, but not HSP60, negatively affected the accumulation of CPMV. Additionally, we have shown that the spread of both tubule-guided (CPMV) and ribonucleoprotein complex dependant (TMV) virus transport is affected by the silencing of either CDC48 or HSP70, suggesting these two proteins affect conserved viral processes. However, further directed studies are required to unravel the exact mode of action of HSP70 and CDC48 proteins in CPMV transport and infection.

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Chapter five

The role of plasmodesmata-located proteins in tubule-guided virus transport is limited to the plasmodesma.

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The role of plasmodesmata-located proteins in tubule-guided virus transport is limited to the plasmodesma.

Abstract

Intercellular spread of plant viruses involves passage of the viral genome or virion through plasmodesma (PD). Some viruses severely modify the PD structure, as they assemble a virion carrying tubule composed of the viral movement protein (MP), inside the PD channel. Successful modulation of the host plant to allow infection, requires an intimate interaction between viral proteins and both structural and regulatory host-proteins. To date, however, very few host proteins are known to positively affect virus spread. Receptor-like plasmodesmata located proteins (PDLPs) have been shown to contribute to tubule formation of both *Cauliflower mosaic virus* and *Grapevine fanleaf virus* in leaves of *Nicotiana benthamiana*. PDLP localisation in PD was shown to be essential for the intercellular spread of the latter virus. In this paper we investigated the role of PDLPs in intercellular transport of another tubule-forming virus, *Cowpea mosaic virus*. The MP of this virus was found to specifically interact with PDLP at PD, as has been shown for the two other tubule-forming viruses. Expression of PDLPs and MPs in protoplasts revealed that these proteins do not co-localise at the site of tubule initiation in the absence of intact PD. Additionally, we showed that tubule assembly in protoplasts does not require an interaction with PDLP at the base of the tubule, as has been observed *in planta*. These results suggest that a physical interaction between MPs and PDLP is not required for assembly of the movement tubule *per se*, nor does it seem to be required for anchoring of the tubule in the plasma membrane. These results further imply that the beneficial role of PDLP in viral movement tubule assembly is confined to the structural context of the PD.

Introduction

Plant viruses spread from the initially infected cells to neighbouring uninfected cells through cell wall spanning channels called plasmodesma (PD, plural plasmodesmata, PDs). Native PDs regulate the transport of macromolecules between cells and do not allow the passage of virions or viral genomes (Kragler, 2013). Therefore, plant viruses encode specialized proteins called movement proteins (MPs) which modify the PD to allow passage of viruses or their genome. Viruses that transport their genomes between cells as mature virions need to modify the structure of the PD pore to accommodate formation of a movement tubule (Schoelz *et al.*, 2011), a process that relies on the collaboration with host proteins (Niehl and Heinlein, 2011; Harries and Ding, 2011). This tubule-guided virus transport is exemplified by icosahedral RNA viruses such as *Cowpea mosaic virus* (CPMV) (van Lent *et al.*, 1990) and *Grapevine fanleaf virus* (GFLV) (Ritzenthaler *et al.*, 1995).

Although the substitution of luminal PD components by a viral movement tubule requires severe structural PD remodelling, very little is known about the host proteins that allow or facilitate the assembly of the movement tubule inside the PD. Proteins such as remorin (Raffaele *et al.*, 2009), class 1 reversibly glycosylated polypeptides (Zavaliev *et al.*, 2010), calreticulin (Chen *et al.*, 2005) and plasmodesmata-located proteins (PDLPs, Thomas *et al.*, 2008) were all found to localize to the PD and shown to interact with viral MPs. However, only for PDLPs a positive regulation of viral transport has been shown, while the function of the other PD proteins negatively affects viral spread.

PDLPs were identified from a proteomic screen of *Arabidopsis thaliana* cell wall proteins (Bayer *et al.*, 2006) and characterized by Thomas and co-workers (2008). They found that PDLPs exclusively localize to the PD when expressed under their native promoter. PDLPs have a typical architecture: a short C-terminal cytoplasmic domain, a transmembrane domain and an extensive extracellular N-terminal domain. Furthermore, all eight arabidopsis PDLP isoforms interact with the MPs of GFLV and *Cauliflower mosaic virus* (CaMV) at the base of the movement tubule constructed in the PD (Amari *et al.*, 2010). The interaction between GFLV MP (2B) and PDLPs was shown to be required for tubule formation, as tubule formation was significantly reduced in a triple PDLP

knockout line of arabidopsis (Amari *et al.*, 2010). Correct localization of PDLP to the PD greatly enhanced tubule formation of GFLV, inhibition of PD localization of PDLP, however, fully blocked 2B localization and tubule formation at the PD (Amari *et al.*, 2011). These authors therefore suggested that PDLPs might serve as a PD recognition site for 2B and anchor the movement tubule in the plasma membrane lining the PD. The topology of PDLPs with both apoplastic and transmembrane domains and a cytoplasmic carboxy-terminus that interacts directly with GFLV movement tubules, supports the proposed function of these proteins in tubule anchoring inside the PD.

To test whether the interaction with PDLPs is a general feature of tubule-forming MPs, we employed fluorescence lifetime imaging techniques to determine whether the MP of CPMV also interacts with PDLPs in the PD. Furthermore, we investigated whether the proposed functions of PDLP, i.e. PD recognition, initiation of MP accumulation and tubule anchoring, are intrinsic properties of these proteins by exploring these functions in protoplasts, plant cells which do not have a cell wall or PDs. Our results show that PDLP interacts with the MP of CPMV *in planta* in a similar fashion as has been described for GFLV and CaMV. In protoplasts, however, MP accumulations did not localise with the PDLP and no PDLP could be detected at the base of the movement tubules formed on the protoplast surface.

Materials and methods

Plant material

Nicotiana benthamiana (Nb) plants were grown on soil in a climate controlled growth chamber at 70% humidity under a long photoperiod regime (16 h light, 8 h dark) at temperatures of 22°C (±1°). Wild type and triple PDLP knockout (PDLP^{-123}) *Arabidopsis thaliana* plants (ecotype Col-0; Amari *et al.*, 2010) were grown under the same conditions at 20°C (±1°).

Constructs

The plasmids containing an N-terminal fusion of GFLV 2B MP to GFP (GFP-2B) and *Arabidopsis thaliana* PDLP1-GFP and PDLP1-RFP were obtained from dr. Khalid Amari and have been described previously (Amari *et al.*, 2010). A fusion of GFP to the C-terminus of CPMV MP was created in the binary vector pSOL2095 (Liebrand *et al.*, 2012). The 48K reading frame from the pMON-MP-GFP vector (Pouwels *et al.*, 2002) was amplified by PCR using Phusion polymerase (Thermo Scientific) and primers containing AttB sites (underlined) to allow subsequent gateway (Invitrogen) cloning (Fw(5′ to 3′): GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGGAAAGCATTATGAGCCG; Rv (5′ to 3′) :GGGGACCACTTTGTACAAGAAAGCTGGGTATTGTGGAAAAGCCACATTC). The amplified fragment was inserted into the pDonor207 vector and the 48K containing pDNOR207 plasmid was recombined with the pSOL2095 binary vector. The sequence of the fusion construct in the pSOL vector was verified. For visualisation of endoplasmic reticulum (ER) a 35S promoter-driven GFP-HDEL construct was used, which expresses GFP with the -HDEL ER retention signal fused to its C-terminus (Haselhoff *et al.*, 1997).

*Agrobacterium tumefaciens-medi*ated transient protein expression in *N. benthamiana*

Transformed *A. tumefaciens* (LBA4404, carrying 48K-GFP, GFP-2B or PDLP1-GFP constructs, and GV3101 carrying the PDLP1-GFP construct) were used at an OD_{600} of 0.5 in an *A. tumefaciens* transient transformation assay (ATTA) performed as previously described by de Ronde *et al.*, (2013). Leaves of 4-5 week-old *N. benthamiana* plants were infiltrated with bacterial suspensions and fluorescent

signals could usually be detected 2 d post ATTA. Co-infiltration of bacterial suspensions containing different constructs was done by mixing the suspensions in a 1:1 ratio. Microscopic analysis of the infiltrated area was done 3 or 4 d post ATTA.

Isolation and transformation of protoplasts

Protoplasts were isolated from young leaves, 4 cm in length 3.5 cm in width (± 0.5 cm), of 3-4 week-old *N. benthamiana* plants. These leaves were cut in a featherlike pattern of 1 mm wide strips from the midvein outward. The leaves were then put with their abaxial side on an enzyme solution to release mesophyll protoplasts, which subsequently were isolated as described by van Bokhoven *et al.* (1993). Introduction of plasmid DNA into the protoplasts was done by PEG-mediated transfection with 5 µg of plasmid (per construct) per 10^5 protoplasts using methods described by van Bokhoven *et al.* (1993). Preparation of *A. thaliana* protoplasts and subsequent DNA transfection was done as follows: arabidopsis leaves (fifth to ninth leaf) were harvested and their abaxial epidermis was removed using the "tape-arabidopsis sandwich method" (Wu *et al.*, 2009). Isolation of protoplasts was done according to the protocol described by Sheen (2002), with some modifications. Leaves from which the abaxial epidermis was removed were incubated on the described enzyme solution containing adjusted amounts of enzymes (1% cellulase, 0.25% macerozym, both R10 by Serva) for 2-3 h at room temperature, while gently swirling. Protoplasts were washed three times in W5 medium, prior to transfection. Per 10^5 protoplasts 10 µg of plasmid DNA was added and mixed for 30 sec prior to the addition of 500 µl 40% PEG solution (PEG Mw 3,350 in 0.2 M (D)-Mannitol with 100 mM $Ca(NO_3)_2$). Protoplasts, DNA and PEG were mixed for 30 sec, diluted with 4.5 ml W5, mixed by inversion and incubated at 25°C for 15 min. After two additional washes, protoplasts were stored in W5 medium with 50 µg/ml gentamicin until inspection at 24 h post transfection.

Confocal microscopy

Infiltrated leaf sections were placed in an imaging chamber filled with perfluorodecalin (Sigma), this chamber consisted of two coverslips sealed by perfluorinated grease (RT15, Fomblin). Protoplasts were imaged by sandwiching

a droplet of suspension between two coverslips spaced 0.5 mm apart. Confocal imaging of leaves and protoplasts was done in a Zeiss LSM 510-META confocal laser scanning microscope using a 63x/1.4 plan-apochromat oil immersion lens. The microscope was operated in multi-channel mode, sequentially exciting GFP (488 nm Argon-laser, 5% laser power) and RFP (543 nm Helium-Neon laser, 30-50% laser power) and their emission was detected at 505-530 nm and 560-615 nm, respectively. Callose was detected by infiltration of leaf material with an aniline blue solution 0.1% (w/v) in 67mM K_2HPO_4, pH 9.0 (Merck).

FRET-FLIM measurements

Förster resonance energy transfer (FRET) is a photo-physical process in which the excited-state energy from a fluorescent donor molecule is transferred non-radiatively to an acceptor molecule. FRET is based on weak dipole–dipole coupling and only occurs if donor and acceptor are in very close proximity (<10 nm, Truong and Ikura, 2001). There are several methods to quantify and visualize FRET. Donor fluorescence lifetime imaging (FLIM) is the most straightforward approach, since the fluorescence lifetime is a concentration-independent property. However, fluorescence lifetimes are sensitive to the environment, which is the basis for FRET-FLIM analysis. Typically, FRET-FLIM experiments consist of measuring donor fluorescence lifetimes (here GFP) in the absence (τD) and presence (τDA) of acceptor molecules (here RFP) resulting in spatially resolved color-coded fluorescence lifetime images. Observation of a decreased donor fluorescence lifetime is used as read-out for molecular interactions (Borst and Visser, 2010; Bücherl *et al.*, 2013).

Time correlated single-photon counting FLIM measurements were done on a Leica SP5X-SMD multi-mode confocal laser scanning microscope using a 63x water immersion 1.2NA lens. GFP/RFP were excited using a white-light laser (WLL; or super continuum laser), which emits a continuous spectrum from 470 to 670 nm, within which any individual excitation wavelength in 1 nm increments can be selected. Confocal imaging was performed using internal filter-free spectral photomultiplier tube detectors. GFP and RFP were sequentially excited using WLL GFP at 488 nm (10% laser power) and RFP at 554 nm (8% laser power). Fluorescence was detected at 505-545 nm for GFP and 560-615 nm wavelengths for RFP. For FRET- FLIM experiments, the WLL (488 nm) at a pulsed frequency of

40 MHz was used. For recording of donor fluorescence, an external fibre output was connected to the Leica SP5 X scan head and coupled to a Hamamatsu HPM-100-40 Hybrid detector (Becker & Hickl), which has a time resolution of 120 ps. Selection of GFP fluorescence was performed using band pass filter 505-545 nm. Images with a frame size of 128 x 128 pixels were acquired with acquisition times of up to 90 sec. From the fluorescence intensity images the decay curves were calculated per pixel and fitted with either a mono- or double-exponential decay model using the SPCImage software (Becker & Hickl, version 3.2.3.0). The mono-exponential model function was applied for donor samples with only GFP present. For samples containing two fluorophores, GFP and RFP, a 2-exponential model function was used without fixing any parameter.
Data was analysed using SPC image and FRET efficiencies were calculated using the equation:

$$\mathrm{E} = \left(\frac{R_0^6}{R_0^6+R}\right) = 1\frac{\tau_{DA}}{\tau_D}$$

where R_o is the Försters radius, R is the distance between donor and acceptor and where **τ**D and **τ**DA are the lifetime of GFP in the absence and presence of RFP-acceptor respectively.

Statistical analysis

To determine whether the decrease in fluorescent lifetime in the presence of an acceptor molecule was statistically significant, the non-normally distributed lifetime data was analysed by Mann–Whitney–Wilcoxon tests, these tests showed a significant (P=< .001) decrease in lifetime of both 48K-GFP and GFP-2B when in the presence of PDLP-RFP acceptor molecule.

Results

Transiently expressed MPs localise to plasmodesma and form tubules *in planta*

To test whether CPMV MP (48K) interacts with PDLPs located in PDs, a C-terminal fusion of GFP to the 48K protein (48K-GFP) was inserted into a binary vector which allows *in planta* expression via ATTA. Confocal microscopy of transformed epidermal cells revealed that most 48K-GFP fluorescence was observed in punctate spots at the cell wall (Figure 1A) and to a lesser extent as fluorescent tubules across the PD (Figure 1B), the latter were visualized by aniline blue staining of callose (Figure 1C,D). Apparently, the C-terminal fusion of GFP to the 48K MP does not hamper its localisation to the PD nor its assembly into tubules, indicating that this construct is functional and suitable for *in planta* experiments. The expression of GFP fusions to PDLP (PDLP-GFP, Figure 1E-H) and GFLV 2B MP (GFP-2B, Figure 1I-L) also resulted in the formation of punctate spots (PDLP and 2B) and cell wall spanning tubules (2B).

The movement protein of CPMV interacts with PDLP at the PD

To establish whether PDLPs and 48K proteins co-localize and interact with each other, a representative member of the arabidopsis PDLP family (PDLP1) was fused to RFP (PDLP-RFP) and transiently expressed along with 48K-GFP in *N. benthamiana* leaves (Figure 2A-C,E-G). As a positive control GFP-2B and PDLP-RFP, two proteins that are known to interact in the PD, were co-expressed (Figure 2I-K,M-O). In transformed leaf cells bright fluorescence of both 48K-GFP and PDLP-RFP could be observed in overlapping spots in the cell wall, showing that these proteins co-localise in the PD (Figure 2E-G). As expected, the expression of both PDLP-RFP and GFP-2B resulted in co-localisation at the base of movement tubules formed in the PD by 2B (Figure 2M-O). To establish whether co-localisation of MPs and PDLP signified an interaction between these proteins, FRET-FLIM experiments with MP-GFP as photon donor molecule and PDLP-RFP as photon acceptor molecule were conducted. As FRET only occurs when donor and acceptor molecules are in close proximity (<10 nm), the transfer of energy between the fluorophores corresponds to a molecular interaction of the fused proteins. FRET efficiency is measured by fluorescence lifetime imaging (FLIM)

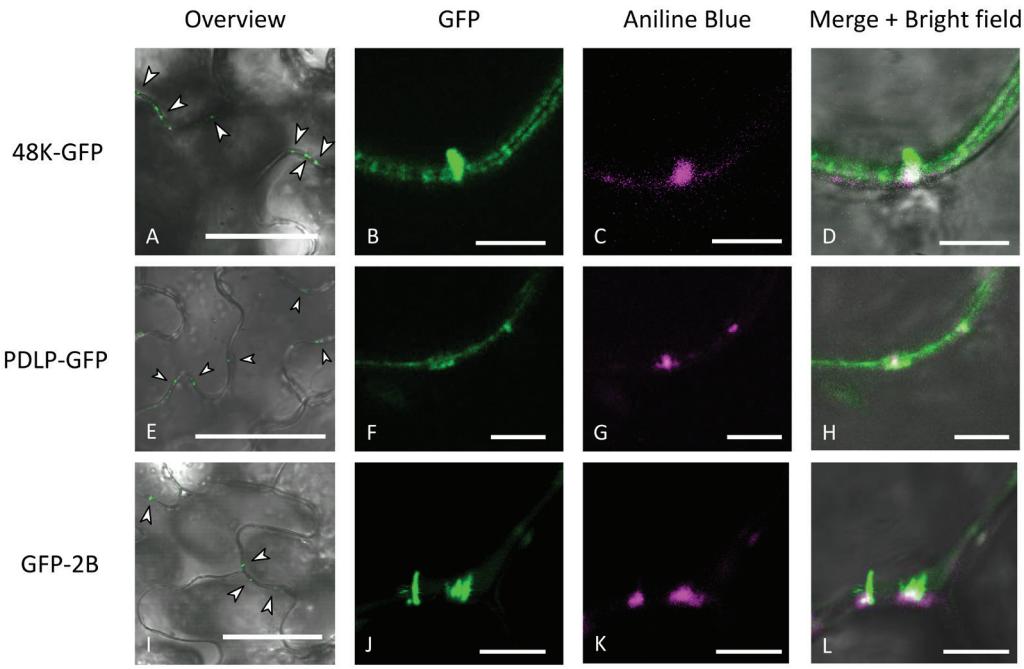

Figure 1: Transiently expressed PDLP and MP localise to PD.
GFP-labelled 48K, PDLP and 2B localise as punctate spots at the cell wall, indicated by arrowheads in the overview panes (**A,E,I**). Detailed confocal images show the movement tubules formed by 48K-GFP (**B-D**) and GFP-2B (**J-L**) localise in plasmodesmata which are identified by callose staining using alinine blue(C,K). Localisation of PDLP-GFP with callose staining confirmed the PD localisation of the MP(**F-H**). Imaging was done three day post ATTA. Scale bars in A, E, I are 50 µm; scale bars in B-D, F-H and J-L are 5 µm.

of the donor molecule, as the fluorescence lifetime of the donor decreases if its energy is transferred to an acceptor molecule. FRET-FLIM measurements showed that co-localisation of either 48K-GFP or GFP-2B with PDLP-RFP (figure 2G and 2O respectively) coincided with a significant decrease in GFP fluorescence lifetime relative to the fluorescence lifetime of individually expressed 48K-GFP and GFP-2B proteins (compare Figures 2D and 2H, and Figures 2L and 2P, respectively), which implies an interaction between PDLP and these MPs. The decrease in donor lifetime for both MPs in the presence of PDLP-RFP is quantified in Figure 3.

Movement proteins do not co-localize with PDLP in protoplasts

To further investigate the interaction between PDLP and MPs, fluorescent protein fusions were expressed in *N. benthamiana* protoplasts. Protoplasts are isolated plant cells devoid of a cell wall and consequently PDs are absent. This allows investigation of intrinsic properties of PDLP independent of the structural context of the PD. Transient expression of 48K or 2B MPs resulted in outgrowth of movement tubules from the protoplast surface (4A,4B). Co-expression of PDLP

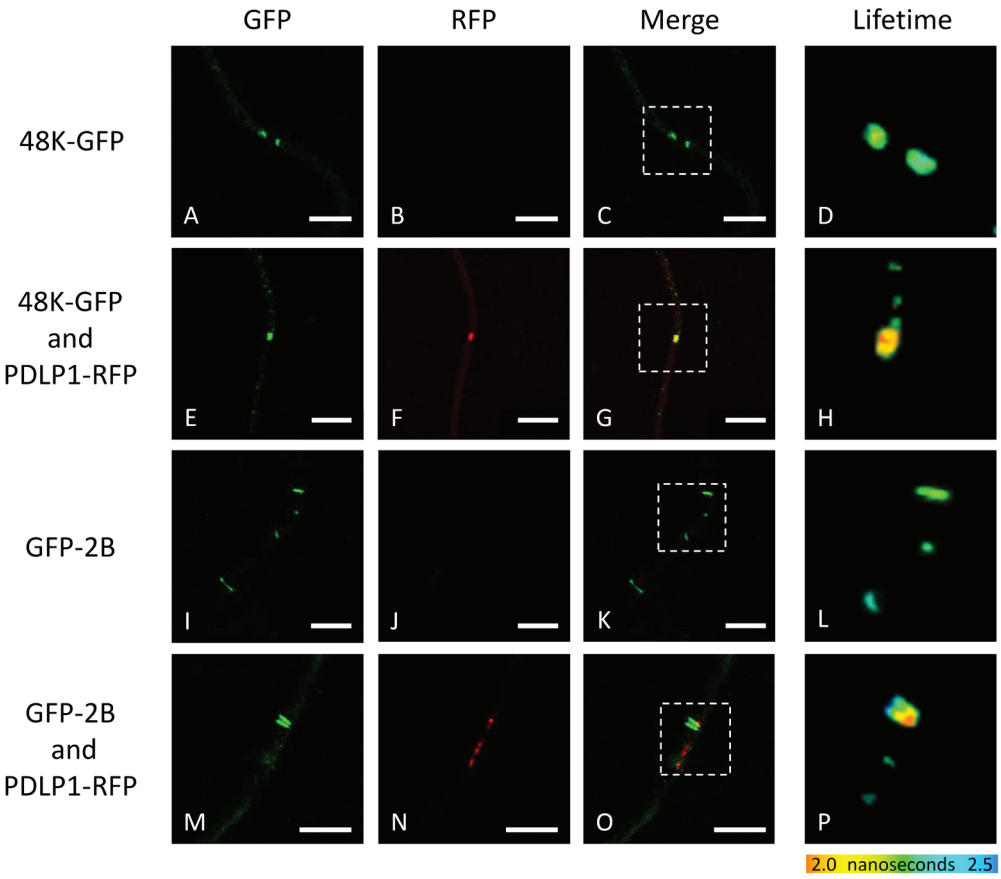

Figure 2: Interactions of 48K-GFP and GFP-2B with PDLP-RFP in PDs.
Confocal images showing the location and fluorescence lifetime of GFP-labelled CPMV MP 48K, either in the absence (**A-D**) or presence of PDLP-RFP (**E-H**). Localisation and lifetime of GFLV MP 2B is also presented in absence (**I-L**) and presence of PDLP-RFP (**M-P**). Reduced fluorescence lifetimes for 48K-GFP in the presence of PDLP-RFP can be seen in H (compare lifetime to D), and GFP-2B in P (compare lifetime to L). Lifetime image panes (left column) display a pseudo-coloured image representing GFP lifetime as indicated by colour scale below the column. White dashed boxes indicate spots portrayed in lifetime image.
Scale bar = 5 µm.

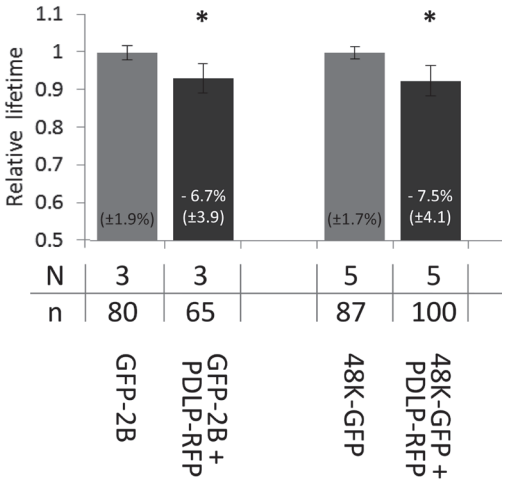

Figure 3: Relative fluorescence lifetime of GFP-MP fusions.
Normalised fluorescence lifetime of GFP photon donor (48K-GFP and GFP-2B fusions) (grey bars), and relative fluorescence lifetime of donors in the presence of the PDLP-RFP photon acceptor (black bar). Percentage of decrease in fluorescence lifetime and standard deviations are displayed numerically in each bar. Error bars display standard deviation.* indicates significant (P>0.05) reduction of donor fluorescence lifetime. N = number of experiments, n = number of fluorescent spots measured.

and MP in protoplasts would reveal whether PDLPs direct the MP accumulation at the plasma membrane and whether anchoring of the movement tubule base to the plasma membrane requires PDLP.

In protoplasts PDLP-GFP formed both peripheral and internal punctate spots (Figure 4C,4D). Expression of the 48K-GFP construct (from either pSOL (Figure 4A) or pMON (Figures 4E-H) vector) or GFP-2B construct (Figure 4B and4 I-L) resulted in formation of peripheral punctate spots and fluorescent tubules protruding from the protoplast surface. Thus all three proteins: 48K-GFP, GFP-2B and PDLP-RFP, localised to peripheral spots. However, co-expression of PDLP-RFP with either 48K-GFP or GFP-2B did not result in co-localisation (Figure 4E-G and 4I-K). Close inspection of the assembled tubules showed that PDLP-RFP was not found at the base of either 48K and 2B tubules (Figure 4H and 4L respectively). Quantification of the co-localisation between PDLP and MPs leads to the conclusion that both 48K-GFP and GFP-2B show almost complete co-localisation with PDLP-RFP in plant cells, however, in protoplast co-localisation was only sporadically observed (Figure 5). The large number of

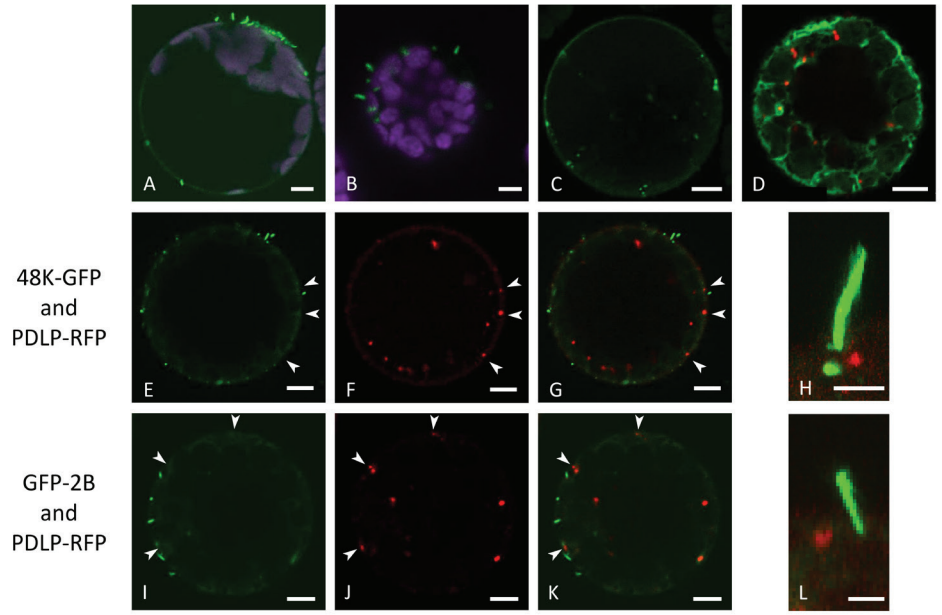

Figure 4: Localization of PDLP and movement proteins in *N.benthamiana* protoplasts. **A)** 48K-GFP expressed from pSOL2095 localizes to peripheral spots and short tubules. **B)** GFP-2B forms protruding tubules when transiently expressed in protoplasts **C)** PDLP-GFP localization to peripheral spots and to a lesser extend in internal spots. **D)** Localization of internal PDLP-RFP spots with ER, the latter is visualized by GFP-HDEL. **E-G, I-K)** Localization of co-expressed GFP labelled MPs (E,I) and PDLP-RFP (F,J) (merge in G and K), arrowheads indicate peripheral PDLP spots. **H, L)** Extended focus image showing fluorescent 48K and 2B movement tubules (H and L respectively) and PDLP-RFP at distinct locations, without overlap at the base of the tubule. Chloroplast auto-fluorescence (A,B) is displayed in magenta. Scale bar A-G and I-K is 5 µm, scale bar in H and L is 2µm.

	Co-localisation (in %)	Standard deviation (in %)
In plasmodesmata		
48K-GFP with PDLP-RFP	98.4	±3.6
GFP-2B with PDLP-RFP	97.2	±5.1
In protoplasts		
48K-GFP with PDLP-RFP	5.4	±7.3
GFP-2B with PDLP-RFP	4.9	±7.4

Figure 5: Quantification of co-localization of MPs with PDLP in plants and protoplast. Levels of co-localisation observed between the CPMV (48K) and GFLV (2B) MPs and PDLP-RFP in the plasmodesmata of plant cells and in protoplasts. Co-localisation levels represent averaged values from three pooled experiments obtained by quantification of at least 15 cells in each experiment.

movement tubules formed on protoplasts despite the scarce occurrence of co-localisation suggests that a PDLP interaction is not essential for tubule formation in protoplasts.

Amari and co-workers (2010) showed that tubule formation of GFLV was severely reduced in a triple PDLP knockout genotype of *Arabidopsis thaliana* (At PDLP^{-123}). As arabidopsis is not a host for CPMV, tubule formation of 48K-GFP could not be tested *in planta*. However, CPMV is able to infect arabidopsis protoplasts, therefore, protoplasts from wild type and from PDLP^{-123} genotype were transfected with 48K-GFP or GFP-2B constructs. In transfected protoplasts from both genotypes tubule formation was observed (Figure 6). Transfection with either 48K-GFP or GFP-2B constructs yielded similar levels of tubule formation in wild type and PDLP^{-123} cells but due to limited transfection efficiency quantification was not possible.

Discussion

PDLP isoforms are exclusively found in PDs. In this structure PDLPs associate with MPs and benefit the assembly of viral movement tubules (Amari *et al.*, 2010). Our data show that PDLP1 co-localizes and interacts with the MPs of both CPMV and GFLV at PDs in *N. benthamiana* (Figure 2). When co-expressed in protoplasts from the same host, however, no co-localisation and hence no interaction between the MPs and PDLPs was observed (Figure 4). In both cases the expressed MPs were competent to form movement tubules, in PDs and at the cell surface of protoplasts. The formation of peripheral punctate spots and tubules in protoplasts and the absence of co-localisation between MPs and PDLP suggests that PDLP are not directly involved in the accumulation of MPs or anchoring of the tubule at in the plasma membrane.These findings also suggest that the interaction between PDLP1 and the MP requires the structural context of the PD.

Transient expression of a fluorescent fusion protein consisting of the CPMV MP and GFP in *N. benthamiana* leaves showed the expected localisation of the MP as peripheral punctate spots at the cellular periphery and to a lesser extend in movement tubules (Figure 1; Gopinath *et al.*, 2003). The punctate spots may

represent short tubules inserted in the PD, as the spots are retained in the cell wall upon plasmolysis (data not shown) and as the 48K-GFP construct effectively forms tubules on protoplast (Figure 4). Thus the expressed CPMV MP is fully competent in the formation of tubules, evenhough the CPMV tubules formed *in planta* are not as obvious as those formed upon expression of GFLV 2B.

Co-localisation experiments in which GFP labelled MPs were expressed with PDLP-RFP *in planta* revealed that CPMV 48K and GFLV 2B specifically localised with PDLP-RFP in the PD. FRET-FLIM analysis showed a significant reduction in 48K-GFP fluorescence lifetime, indicating that 48K interacts with PDLP at the PD (Figure 2). Interaction between 2B and PDLP was also observed in the PD, which is in line with previous reports (Amari *et al.*, 2010, 2011). The FRET-efficiencies of the MP-PDLP interactions were highly similar for both 48K and 2B (7.5% and 6.7% respectively, Figure 3), which suggests that the association of these movement proteins with PDLP occurs in a similar fashion. Whether this interaction is required for CPMV MP tubule formation in PD, as has been shown for the MP of GFLV, remains to be established. Expression of 48K and 2B in protoplasts of PDLP triple knockout arabidopsis plants resulted in tubule formation with an abundance and time frame similar to that observed in wild type protoplasts (Figure 6). Although our limited dataset does not allow detailed quantitative analysis, the presence of movement tubules in the first place implies that knockout of three PDLPs does not severely hamper tubule formation, if at all, by either 48K or 2B MP in protoplasts. The pertinent experiments require

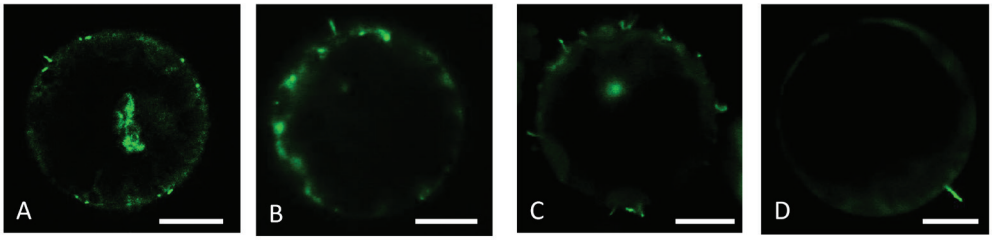

Figure 6: Tubule formation of 48K-GFP and GFP-2B in arabidopsis protoplasts.
A,B) Wild type *Arabidopsis thaliana* protoplasts transiently expressing 48K-GFP (A) or GFP-2B (B) show the outgrowth of fluorescent movement tubules. **C,D)** Triple PDLP knockout (PDLP^{-123}) *A. thaliana* protoplasts expressing 48K-GFP (C) or GFP-2B (D) proteins also showing the outgrowth of viral movement tubules. Scale bar = 10 µm

repetitions in protoplasts and tissues of PDLP silenced plants that do host CPMV infection, to obtain robust data on the influence of PDLP on the tubule-forming capacity of 48K in protoplast and in PDs. However, no PDLP knockdown lines of any CPMV host plants are currently available.

The interaction of 48K with PDLPs supports the hypothesis that the interaction between MPs and PDLPs is conserved among tubule-forming viruses (Amari *et al.*, 2010). Testing whether the movement proteins of viruses such as *Tomato spotted wilt virus (Bunyaviridae)* or *Alfalfa mosaic virus (Bromoviridae)*, which form structurally distinct tubules (Storms *et al.*, 1995; Kasteel *et al.*, 1997), also interact with PDLP in the PD would be very interesting, as this would support the suggested evolutionary relationships between tubule-forming viruses (Melcher, 2000).

Although it is clear that PDLPs interact with MPs of GFLV, CPMV and CaMV in the PD (this work; Amari *et al.*, 2010), the significance and underlying mechanisms of this interaction remain unclear. Because the presence of PDLP in PDs was found to be required for localisation of GFLV 2B to the PD and to enable its tubule formation, Amari *et al.* (2011) suggested that PDLP family members facilitate the accumulation of MPs at PD and anchoring of movement tubules to the plasma membrane. We have tested this hypothesis by co-localisation studies of PDLP and viral MPs in protoplasts. As PD are absent from such cells, we could investigate whether PDLPs serve as recognition and/or anchoring signals for MPs when not associated with PD. PDLP and GFLV MP showed an interaction *in planta* even after the movement tubule was formed, so we assumed that this continued interaction would result in substantial co-localisation of these proteins in protoplasts. However, in protoplasts no obvious co-localisation was observed between PDLP and either of the MPs (Figure 5). This suggests that PDLPs do not serve as recognition/retention signal for MP accumulation. Because tubules originate from peripheral spots (Pouwels *et al.*, 2004) we propose that PDLPs do not serve as a catalyst for tubule initiation in protoplasts, as this function would require co-localisation of these proteins, which is not the case.

A possible explanation for the lack of PDLP-directed accumulation of MPs in protoplasts, in contrast to the situation *in planta*, could be the absence of a PD-specific complex composed of various PD-proteins including PDLPs. The recognition of such a complex by MPs could depend on an interaction with one

of the other (currently unknown) proteins, or could depend on the structural organization of this multi-protein complex inside the PD. The absence of PDLP at the base of the 48K and 2B movement tubules formed on *N. benthamiana* protoplasts, indicates that membrane anchoring of the movement tubule, required for directed tubule outgrowth, is not mediated by an interaction with PDLP. It is likely that another conserved protein is required for the anchoring of the movement tubule to the plasma membrane, as expression of 48K in insect cells results in tubule formation (Kasteel *et al.*, 1996) and no proteins with the DUF26 domain, characteristic for PDLPs, were found in insect protein databases (protein-BLAST, NCBI.com).

The exact function of PDLP in virus movement remains to be established, however, our studies have shown that PDLP1 interacts with the MP of CPMV in a manner similar to the previously established interaction with GFLV and possibly CaMV (Amari *et al.*, 2010). In addition we have shown that in protoplasts PDLP are not required for localisation or accumulation of MPs prior to tubule outgrowth and that the plasma membrane anchoring of movement tubules is not mediated by PDLP. These new insights emphasize the importance of the structural environment of the PD in the analysis of host protein involvement in plant virus intercellular movement.

Acknowledgements

The authors would like to thank dr. Khalid Amari and dr. Christophe Ritzenthaler for kindly providing GFP-2B, PDLP-GFP and PDLP-RFP plasmids, and the triple PDLP knockout *Arabidopsis thaliana* seeds. We would also like to thank Rik Huisman for providing the 35S::GFP-HDEL construct. Hanke Bloksma is kindly acknowledged for her practical assistance. We would like to thank Professor Just Vlak for critical reading of the manuscript.

Chapter six

General discussion.

General discussion

Successful viral infection requires spread of the virus from the initially infected cell to surrounding cells and tissues. The plant virus *Cowpea mosaic virus* (CPMV) spreads from infected cells by assembly of a tubular structure inside plasmodesmata (PD), which forms the conduit for virus particle passage across the cell wall. The aim of the research presented in this thesis was to identify and characterize host proteins involved in this tubule-guided intercellular transport of CPMV. The particular focus has been on the events in intercellular transport that occur at the plasmodesma. These events include: the accumulation of movement proteins (MPs) at the PD, the modification of the PD structure, and the subsequent assembly of the movement tubule inside the PD.

The identification and characterisation of host proteins in CPMV infection.

At the start of this PhD research two host proteins were thought to interact with the CPMV MP. In a column-binding assay purified MPs were immobilized to allow interaction with host proteins from purified cowpea (*Vigna unguiculata*) plasma membrane fractions. Analysis of these attached proteins resulted in the identification of a plasma membrane intrinsic protein 1(PIP1)-like protein and a V-ATPase protein (Carvalho, 2003). Because the PD between plant cells is lined with plasma membrane, it is conceivable that interactions between the MP and plasma membrane proteins, like PIP1, are required for anchoring of the movement tubule in the PD. Movement tubules formed on the surface of CPMV-infected protoplasts are highly similar to the tubules observed *in planta* (Figure 1) and these tubules are also surrounded by the plasma membrane (van Lent *et al.*, 1991). This suggests that interactions between the movement tubule and components of the plasma membrane are also present in this cell system. The interaction between PIP1 and the CPMV MP was further investigated in *Nicotiana benthamiana* protoplasts. However, the NbPIP1-1 protein did not co-localise with the MP and consequently did not interact with this protein at the plasma membrane of either *N. benthamiana* (Chapter 2) or cowpea protoplasts (data not shown). Furthermore, silencing of multiple *Nicotiana* PIP1 isoforms did not inhibit the formation of movement tubules in infected protoplasts. These findings, combined with the observation that PIP1 isoforms are abundant in the plasma

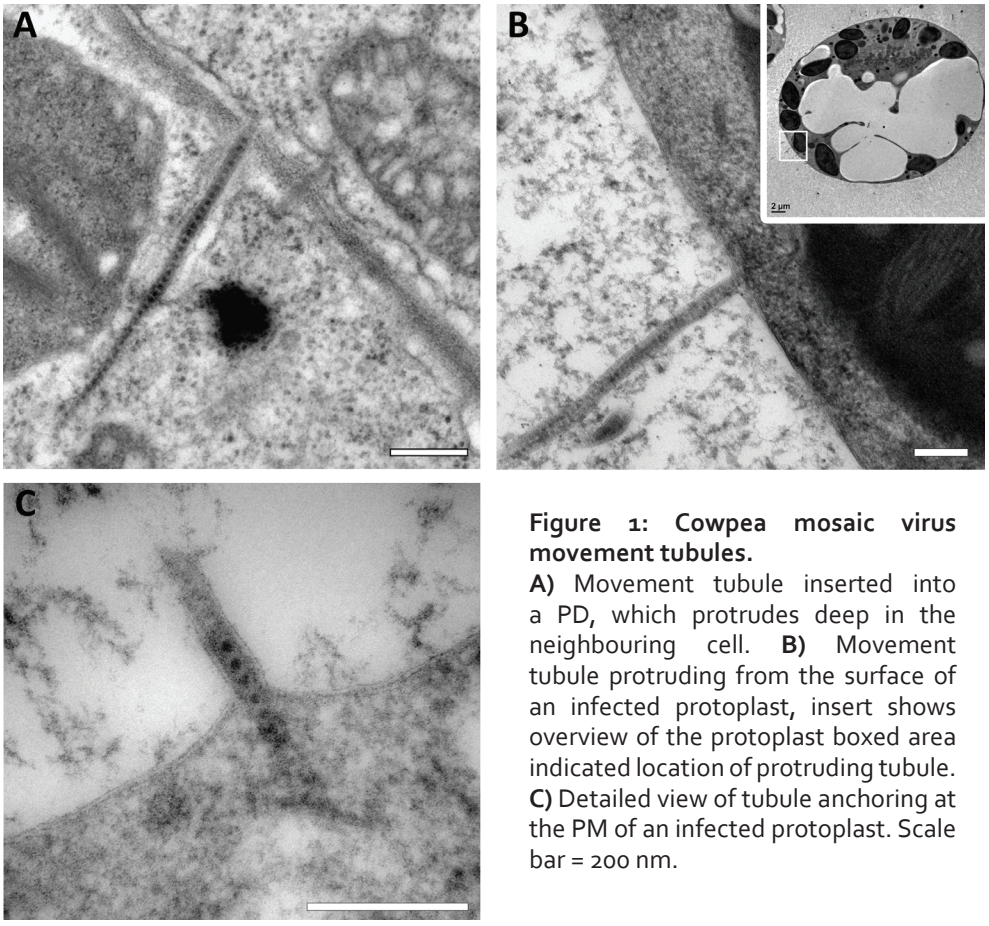

Figure 1: Cowpea mosaic virus movement tubules.
A) Movement tubule inserted into a PD, which protrudes deep in the neighbouring cell. **B)** Movement tubule protruding from the surface of an infected protoplast, insert shows overview of the protoplast boxed area indicated location of protruding tubule. **C)** Detailed view of tubule anchoring at the PM of an infected protoplast. Scale bar = 200 nm.

membrane (Kammerloher *et al.*, 1994; Johanson *et al.*, 2001) but were absent in our proteomic analysis of the plasma membrane-movement tubule complex (Chapter 3), suggest that PIP1 proteins do not have a function in tubule-guided movement and probably were 'false positives' in the study of Carvalho (2003).

To identify host components that associate with the movement tubule, a proteomic analysis of isolated tubules was performed (Chapter 3). Movement tubules formed on the surface of protoplast are abundant and engulfed in the plasma membrane, these tubules are also far more accessible for isolation than tubular structures embedded in the PD. Therefore movement tubules from infected cowpea protoplasts were used as input for this proteomic analysis. The isolated membrane-tubule complexes were treated with a mild detergent to allow anti-MP antibodies to bind the tubule in the subsequent

immunoprecipitation procedure. Furthermore, this detergent treatment was expected to partially dissolve the plasma membrane. This would cause the release of non-interacting plasma membrane proteins, while retaining those that truly interacted with the movement tubule, thus reducing the number of false positive interactions. Due to the limited information on the genomic and proteomic composition of cowpea, the majority of peptides identified by mass-spectrometry could not be assigned to specific proteins. However, by composing a custom protein-sequence database containing all known protein sequences from members of the taxonomic order *Fabales* (which includes cowpea and sequenced organisms such as barrel medic (*Medicago truncatula*), and soybean (*Glycine max*)) it was possible to identify 117 host proteins in the mass-spectrometry input. Using this heterologous protein database approximately 23% of the peptides could be annotated. If it would have been possible to use *Arabidopsis thaliana* in this proteomic analysis, of which the genome is known, the fraction of resolved peptides would probably have been much higher which could in turn have resulted in the identification of more host proteins. Even though CPMV readily infects *Arabidopsis thaliana* protoplasts, it proved impossible to isolate and infect the amount of arabidopsis protoplasts needed to obtain sufficient tubular material for the experiments described. Further analysis of the proteomic harvest of tubules from infected cowpea protoplasts would require the sequencing of the genome of this plant.

Seven of the 117 host proteins identified in the proteomic analysis were found to be candidate components of the membrane-tubule complex (Chapter 3). Two heat shock protein (HSP) 60s and the "ATPase-associated with diverse cellular activities", or AAA-protein, were selected for further characterization, along with a HSP70 (Chapter 4). The involvement of these proteins in CPMV infection was tested by virus-induced gene silencing (VIGS) assays in *N. benthamiana*. The VIGS approach showed that silencing of either HSP70 or the CDC48 class AAA-protein affected the accumulation and spread of CPMV and also affected the spread of *Tobacco mosaic virus* (TMV). This suggests a functional role for these host proteins in the viral infection cycle (Chapter 4), although secondary effects as a consequence of affected basic metabolic processes cannot be excluded.

As the silencing experiment targeted conserved sequences, it was not possible to pinpoint the specific *N. benthamiana* protein isoform that associated with the

MP and affected CPMV and TMV infection. In future work, thorough investigation of the *N. benthamiana* genome could identify the HSP70 and CDC48 isoforms present. A subsequent *in planta* screening with fluorescently labelled HSP70 and CDC48 isoforms could then identify whether these proteins localise to the site of tubule assembly. Additionally, co-expression of these proteins with an appropriately labelled fluorescent MP and FRET/FLIM measurements (Borst and Visser, 2010) could show, whether these proteins interact with the MP. The fact that spread of both CPMV and TMV were negatively affected by silencing of CDC48 and HSP70 may suggests that these proteins chaperone fundamental steps in cell-to-cell movement and therefore testing their interaction with MPs should not be limited to tubule-forming MPs.

Heat shock protein 70

The assembly of a virion-containing movement tubule inside the PD requires the multimerization of MPs while simultaneously incorporating virus particles. The correct assembly of multi-protein complexes can be assisted by chaperone proteins, such as HSP70 and its co-chaperones (Mayer, 2013; Saibil, 2013). Both HSP70 and its ancillary co-chaperones are thought to interact with viral MPs (Hofius *et al.*, 2007; von Bargen *et al.*, 2009; Shimizu *et al.*, 2009; Krenz *et al.*, 2010) and have been found to chaperone the assembly of viral protein complexes (Mine *et al.*, 2012; Wang *et al.*, 2009). It is therefore possible that the association between HSP70 and viral MPs assists in the assembly of movement complexes at the PD. Alternatively, HSP70 might be required inside the PD, as these proteins are able to travel though PDs (Aoki *et al.*, 2002) and are known to facilitate the intercellular movement of a *Closterovirus* (Alzhanova *et al.*, 2001), a *Geminivirus* (Krenz *et al.*, 2010), a *Potyvirus* (Hofius *et al.*, 2007) and endogenous proteins (Aoki *et al.*, 2002). The *Closterovirus*-encoded HSP70 homologue governs the interaction between the virion and the PD. Its motor domain is thought to drive the insertion of the filamentous *Closterovirus* virion into the PD by the hydrolysis of ATP, because mutations in the ATP hydrolysing domain inhibited intercellular spread of this virus (Alzhanova *et al.*, 2001). The identification of a HSP70 isoform in the membrane-tubule complex, together with the reported interactions between viruses and HSP70 and their co-chaperones (Alzhanova *et al.*, 2001; Hofius *et al.*, 2007; von Bargen *et al.*, 2009; Shimizu *et al.*, 2009; Krenz *et al.*,

2010; Mathioudakis *et al*,. 2012) suggest that HSP70-assisted processes might be a general requirement for intercellular transport. HSP70 located at the PD neck can facilitate the trafficking of viral movement structures, i.e. movement tubules, virions or ribonucleoprotein complexes, into and across the PD by the generation of motive forces supplied by its motor domain. This hypothesis would explain our results showing that silencing of HSP70 retards the spread of both CPMV and TMV. A study into the *in vivo* localisation of the different HSP70 isoforms would be required to test this hypothesis.

Cell-division-protein 48

The cell-division-protein 48 (CDC48) AAA-proteins have been found in two independent studies on virus intercellular transport (Chapter 3 and 4; Niehl *et al.*, 2012). In both studies the CDC48 proteins associated with the MP at the MP-membrane interface and both studies showed that the expression levels of CDC48 affected virus spread, which underscores the potential role of CDC48 in virus intercellular transport. The CDC48 proteins have been suggested to regulate the proteolysis of excessive and misfolded MP, which in turn regulates the switch between TMV replication and intercellular transport (Niehl *et al.*, 2013). Whether the CDC48 proteins also fulfil such a function in CPMV infection is currently unknown. It is possible that the removal of misfolded MPs results in enhancement of the assembly of CPMV movement tubules, which is in accordance with the results showing that silencing of CDC48 resulted in reduced CPMV spread. Whether misfolded MP is extracted from the ER or from another membrane needs to be established, as the ER seems dispensable in CPMV infection (Pouwels, 2002). It is therefore more likely that CDC48 proteins extract misfolded proteins from the protein complexes, such as peripheral punctate spots or assembling tubules. The localisation of CDC48 proteins during CPMV infection will undoubtedly provide further insight in the role of CDC48 in tubule-guided cell-cell movement, but does require the identification of the *N. benthamiana* CDC48 isoforms. Further understanding of the localisation and functionality of these proteins may provide insights in the overlap between the different pathways for intercellular movement illustrated by CPMV (via virion-carrying movement tubules inserted into modified PD) and TMV (by diffusion of viral ribonucleoprotein complexes along the desmotubule), as the spread of both

viruses is influenced by silencing of CDC48 proteins (Chapter 4).

Plasmodesmata-located proteins

Investigations into the role of plasmodesmata-located proteins (PDLPs; Thomas *et al.*, 2008) in intercellular movement of CPMV showed that PDLPs interact with CPMV MP present in the PD in a manner very similar to that shown for *Grapevine fanleaf virus* (GFLV) and *Cauliflower mosaic virus* (CaMV) (Amari *et al.*, 2010). However, in protoplasts the expression of the MP of either CPMV or GFLV resulted in tubule formation, but neither of the MPs co-localised with PDLP. Consequently, it was concluded that MPs and PDLP do not interact in these isolated cells, which are devoid of cell wall and PD. These observations suggest that PDLP proteins are not required for MP accumulation at the plasma membrane and subsequent formation of movement tubules, and that the role of PDLPs in virus transport is most likely restricted to the PD. This may also explain the absence of PDLP from our proteomic analysis of the membrane-tubule complex (Chapter 3).

The results of the described protoplast studies (Chapter 5) do not oppose the reported role of PDLP in the PD, where the presence of PDLP is required for correct localisation and tubule formation of the GFLV MP (Amari *et al.*, 2011). These results do, however, imply that localisation of MP to the PD does not depend on a straightforward recognition of individual PDLP isoforms, as this would have occurred also in protoplasts. The conclusion that PDLPs are not required for tubule assembly is supported by various reports that tubules can be formed in insect cells that express plant virus MP (Kasteel *et al.*, 1997; Storms *et al.*, 1995; Liu *et al.*, 2011; Jia *et al.*, 2014), while PDLP homologous genes are absent from the insect genome. Recently, it was shown that virus-encoded tubules also facilitate the transport of plant *Reovirus* particles (*Southern rice black streaked dwarf virus*) across the basal lamina from epithelium to visceral muscle tissues in its insect vector, the white-backed plant hopper (*Sogatella furcifera*) (Jia *et al.*, 2014). This finding emphasizes the importance of studying tubule formation in insect (midgut) cells and shows that host components required for the outgrowth of movement tubules are conserved between plant and animal kingdoms.

It is possible that the recognition of the PDs by MPs is dependent on recognition

of specialized membrane micro-domains found in the PD lumen and at the PD orifice (Raffaele *et al.*, 2009; Faulkner and Maule, 2011; Tilsner *et al.*, 2011). Remorin, a characteristic micro-domain protein, interacts with a MP of Potato virus X (Raffaele *et al.*, 2009), and the localisation of PDLP5 to the central region of the PD implies that it likely associates with membrane micro-domains (Gonzalez-Solis *et al.*, 2014). It is therefore possible that the recognition of these PD specific membrane domains is a prerequisite for the interaction between PDLP and MPs. An association of PDLPs with membrane micro-domains such as lipid rafts or tetraspanin-enriched micro-domains may indicate their importance in regulation of the structural features of the PD. These micro-domains are enriched in sterols, GPI-anchored proteins and sphingolipids that are able to deform and re-shape membranes (Naulin *et al.*, 2014). Analysis of the distinct plasma membrane domains in the PD in relation to virus intercellular movement could resolve the modes of PD recognition by MPs and possibly the anchoring of MP at the plasma membrane. This analysis can be done by studying viral spread in host plants with mutations in their lipid metabolism or their lipid synthesis pathways. An example of such an analysis is the recent study by Peiro *et al.* (2014) showing that changes in the expression levels of patellin proteins 3 and 6 involved in lipid metabolism and lipid-mediated regulatory functions affected the intercellular movement of *Alfalfa mosaic virus.*

The observations that the PDLPs i) remain associated with the PD even after the severe structural modifications by tubule-forming MPs, ii) are present along the length of the PD channel (Maule *et al.*, 2011) and iii) enhance the assembly of viral movement tubules in the PD (Amari *et al.*, 2010) suggest that PDLPs may serve as a tubule-guiding structure inside the PD. Although there is no direct evidence for the existence of such a PDLP structure in the PD, our preliminary data indicate that PDLP1 has a strong tendency to self-interact (Figure 2), implying that multimeric PDLP structures can be formed. Also, the requirement for a structural PDLP component localized in the PD would explain why in protoplasts MPs do not interact with PDLP. Definite proof for such a structure should come from *in situ* visualization of such PDLP structures, a challenging venture for correlative microscopy. Advanced electron microscopic techniques, such as electron tomography, have enabled the visualisation of molecular structures in high definition, but the cell wall and the small size of the PD hinder imaging

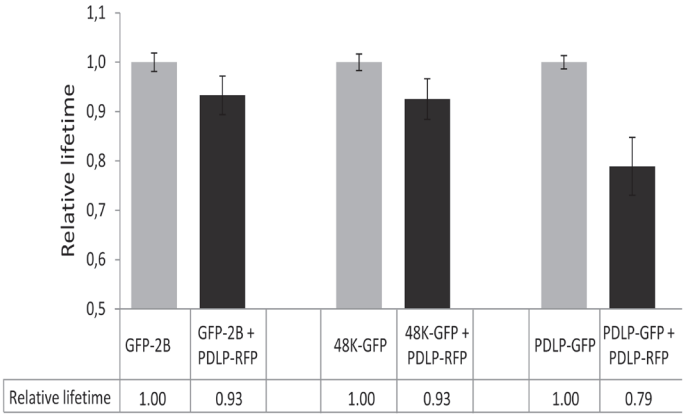

Figure 2: Försters resonance energy transfer (FRET) efficiencies.
GFP (donor) fluorescence lifetime, when expressed alone (grey bar) and its relative lifetime when expressed in the presence of an RFP (acceptor) molecule (black bar) inside plasmodesmata. Showing near identical FRET efficiency for the MPs of CPMV and GFLV (48K and 2B respectively), and a more efficient FRET interaction of PDLP1-GFP with PDLP1-RFP (one experiment, 22 and 23 samples measured). Error bars indicate standard deviation.

of PD substructures in detail (Bell and Oparka, 2011; Faulkner and Maule, 2011). The determination of the localisation of PDLP members in native and tubule-containing PD will, however, illustrate how these PD-proteins are re-organised to allow movement tubule insertion in the PD.

The value of protoplast studies in the analysis of tubule-guided spread of viruses

The differences in the interaction of PDLP and MPs in intact cells and in protoplasts illustrates that, even though highly similar movement tubules are formed in both systems, protoplasts can only provide insight in certain aspects of intercellular virus movement. Protoplasts are a valuable system to study the mechanisms, dynamics and host proteins required for tubule initiation, tubule assembly and the growth of tubules. However, interactions observed in this experimental system should be verified *in planta*, because certain viruses which are not known to form tubules in infected plants are able to form tubules in protoplasts (TMV, Heinlein *et al.*, 1995; *Cucumber Mosaic Virus*, Canto and Palukaitis, 1999; *Apple chlorotic leaf spot virus,* Satoh *et al.*, 2000; *Grapevine berry inner necrosis virus*, Isogai *et al.*, 2003; *Grapevine virus A* and *B*, Haviv *et al.*,

2012) suggesting that multimerization, i.e. tubule formation, is a general feature of MPs from the 30K protein family, and suggesting that interactions with host proteins (possibly at the PD) regulates the mode of intercellular transport in plants. *In planta* studies are therefore essential for the understanding of the involvement of host proteins in other aspects of cell-cell movement such as the induction of the elaborate structural modifications of the PD and the cellular components that are required for assembly of a movement tubule inside the PD.
Using electron microscopy, protoplasts were extensively surveyed for the presence of peripheral punctate spots, local MP accumulations from which the movement tubule outgrowth is initiated (Pouwels *et al.*, 2004). Although readily observed in protoplasts using light microscopy, it is questionable whether these structures also exist in plant cells and/or whether the formation of these structures always precedes tubule initiation. Despite numerous efforts to unravel the ultra-structure of these spots we have been unable to locate them in electron microscopical studies. In immuno-gold labelling studies using anti-MP antibodies the plasma membrane of infected protoplasts was occasionally labelled, however, at the gold-labelled sites no defined structural features could be discerned that resembled the initiation site of tubule formation. The existence of these structures *in planta*, their composition, and their relevance in intercellular spread of CPMV therefore remains to be proven.

A model for host-protein involvement in tubule-guided transport of CPMV

Two studies have been performed to identify host components that interact with the CPMV MP. First, Carvalho (2003) used an *in vitro* approach and identified two candidate host proteins for interaction with the MP. The interaction of one of these proteins (PIP1) was investigated further but could not be verified. Secondly, the mass-spectrometry analysis of highly purified movement tubules from protoplasts (chapter 3) showed seven proteins from five protein families that associated with the CPMV tubule-plasma membrane complex. Subsequent VIGS assays targeting a selection of these proteins provided strong indications for a role of HSP70 and a CDC48 protein in CPMV infection and spread. Studies on interactions of CPMV MP with PDLPs showed that PDLP1 specifically interacts with the MP in PD in plant cells *in planta* but not in protoplasts.
Based on these findings and data from the literature, a tentative model for

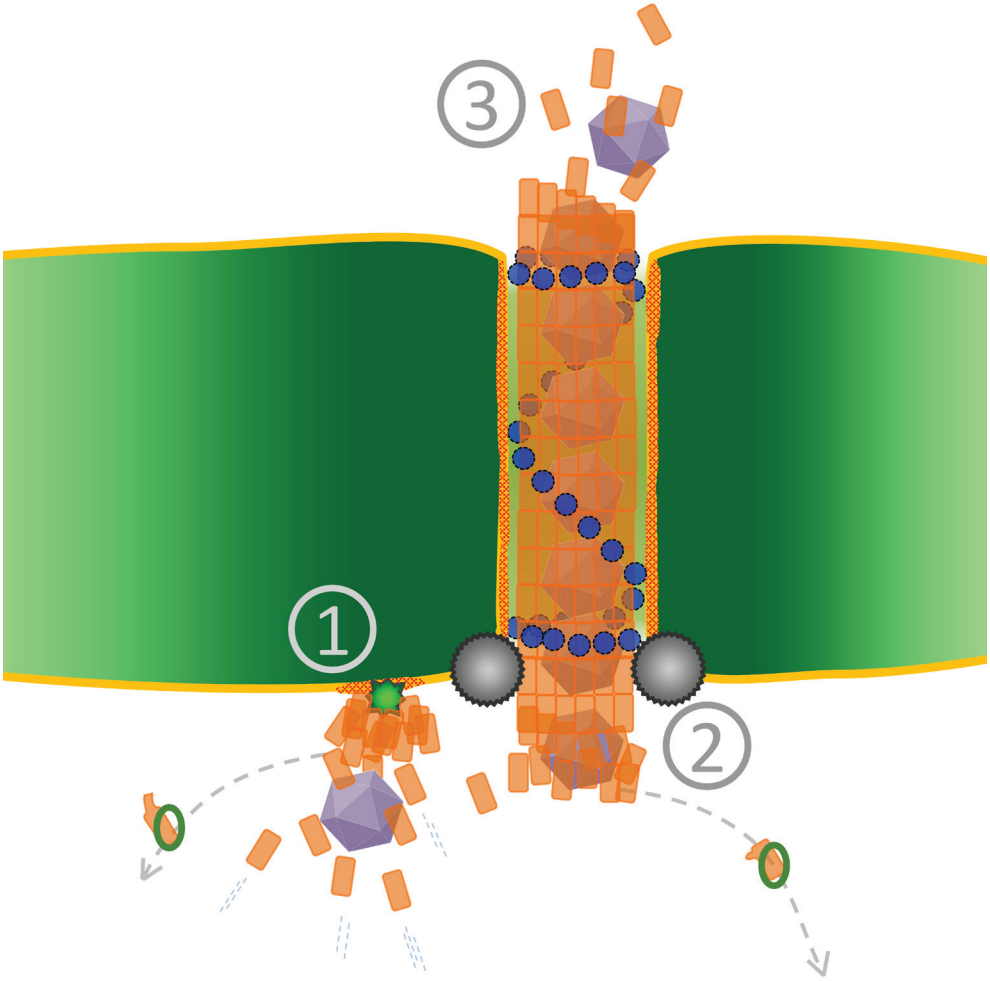

Figure 3: Tentative model for CPMV tubule formation.
The MPs (orange) and virions (purple) accumulate at the plasma membrane (**#1**) as a result of recognition of an unknown protein (green) or membrane domain (hatched in red). The MPs assemble into a movement tubule which encapsulates the virions (**#2**) and is driven into the modified PD orifice by an ATP-driven force generated by HSP70 (grey shapes). Inside the PD, PDLPs (blue spheres) interact with the tubule especially at the PD orifice. In the adjacent cell, the virions are released from the tubule by destabilisation of the tubule by GTP hydrolysis (**#3**). CDC48 proteins (green circles) are suspected to extract of misfolded MP to induce their proteolysis, thereby enhancing the efficiency of cell-cell transport of CPMV.

tubule-guided intercellular transport of CPMV in plant cells is presented in Figure 3. In this model the MP is synthesised near the nucleus and accumulates in a localised spot at the plasma membrane (#1 in Figure 3) by diffusion across the cell (Pouwels *et al.*, 2002). This MP accumulation is likely initiated by recognition of a (unknown) PD-associated plasma membrane protein or a specific membrane domain. Inside the PD the MPs assemble into tubules, while simultaneously incorporating virus particles, likely though binding between the MP and the large coat protein (Carvalho *et al.*, 2003). CDC48-type AAA-proteins extract misfolded MPs to promote their proteolysis, which enhances the efficiency of tubule assembly by selecting for correctly folded MPs. The tubule likely expands by addition of MP/virion complexes to the basal end. This method of elongation is indicated by the protrusion of the movement tubule into the cytoplasm of infected protoplasts (Figure 1C). The growing tubule is inserted into the modified PD by ATP-driven motive force generated by HSP70 (#2 in Figure 3). The establishment and anchoring of the movement tubule in the PD is enhanced by the presence of PDLPs, which form a guiding structure inside the PD channel. When the movement tubule reaches the neighbouring uninfected cell it disintegrates, possibly by destabilisation through the conversion of GTP that is bound by the MP (#3 in Figure 3; Carvalho *et al.*, 2003). Upon release of the virions the infection process starts in the neighbouring cell.

Although multiple host proteins are portrayed in this proposed model, the involvement of host proteins in the tubule guided transport of viruses is expected to extend beyond these examples. Resolving the data generated in the mass-spectrometry analysis could verify and extend the range of tubule-associated host proteins. To exploit the full potential of the proteomic data-set the genome of cowpea would need to be sequenced and annotated.

Studies on virus-host interactomics greatly benefit from host plants with a known genetic background, as this eases the identification and characterisation of the host proteins involved in processes such as the intercellular transport of viruses. The annotation of the *Nicotiana benthamiana* genome improves on a daily basis (see the BTI *N. benthamiana* website) and the growing catalogue of molecular tools available for this plant, make *N. benthamiana* the preferred host for studies on *Cowpea mosaic virus*. Even though the molecular tools available for the model plant *Arabidopsis thaliana* outnumber those of *N. benthamiana*, the range of

pathogens that are able to infect the latter species is far greater, which makes *N. benthamiana* a valuable tool for plant virologists.

The recent report on the role of tubular structures in the intercellular transport of a plant virus in its insect host (Jia *et al.*, 2014), shows that the use of insect cells to study tubule formation by plant viruses is not only feasible, but might also be biologically relevant. Because the tubules assembled in insect cells closely resemble their counterparts in plant cells, insect cell systems could be exploited to identify which host-proteins common to plants and insects are essential for tubule assembly. Because insect cells are easier to maintain and to experiment with than protoplasts, the use of this system should be considered seriously for future studies on the mechanistic aspects of tubule assembly and the requirements of host proteins in tubule initiation.

Large genetic and proteomic screening techniques are becoming more affordable and more frequently applied. Studies on virus-host interactions are therefore able to shift their focus from identifying candidate interacting host components to characterising their role in infection. The real challenge in the study of tubule-guided movement of plant viruses remains to uncover the relation between candidate host proteins and viral proteins and the translation of these relations to the plasmodesmata delimited situation between plant cells.

Appendices

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Summary

The spread of plant viruses from the initially infected cell to neighbouring uninfected cells requires the action of virus-encoded movement proteins (MPs). These MPs modify the plasmodesma (PD), complex pores in the cell wall that interconnect adjacent cells to allow transport of viral genomes between these cells. The PD is the only conduit for communication between plant cells and connects the endoplasmic reticulum, the plasma membrane and the cytoplasm of neighbouring cells. *Cowpea mosaic virus* (CPMV) moves between cells in the form of mature virus particles through a 'movement tubule' assembled inside the PD from MPs. The assembly of this tubule also requires structural remodelling of the PD channel i.e. removal of the interconnecting ER (desmotubule) and proteins from the PD lumen. These modifications inevitably depend on the concerted action of proteins from the virus and host. The aim of the presented research was to identify and characterise host proteins that are required for tubule-guided intercellular movement of plant viruses.

Because the PD channel is lined with plasma membrane the assembly of movement tubules occurs in close proximity to this membrane and the host proteins it contains. In an earlier study, Carvalho (2003) reported an *in vitro* binding between CPMV MP and a plasma membrane intrinsic protein 1 (PIP1). In this thesis the role of PIP1 in virus movement was further investigated. CPMV MP and a PIP1 isoform from the experimental host plant *Nicotiana benthamiana* were labelled with a fluorescent tag and the location and possible co-localisation of these proteins in protoplasts was studied (**Chapter 2**). These experiments showed that *N. benthamiana* PIP1, in contrast to what was found previously, did not co-localise with the MP and hence these proteins do not likely interact. Furthermore, infection of *Nicotiana* protoplasts in which genes for multiple PIP1 isoforms were silenced did not show altered tubule formation, suggesting that tubule formation does not depend on PIP1 activity.

Protoplasts, isolated plant cells without a cell wall and thus without PD, can be infected with CPMV. In these infected protoplasts movement tubules are formed which protrude into the culture medium tightly engulfed by plasma membrane. These plasma membrane-movement tubule complexes were collected to identify the host and viral proteins that are present in this complex (**Chapter 3**). These

complexes were isolated from protoplasts and purified by immunoprecipitation targeting the MP prior to their analysis by tandem mass-spectrometry. This proteomic analysis resulted in the identification of numerous host proteins of which seven were highly abundant in the isolated complexes. These seven proteins were therefore considered candidate components of the membrane-tubule complex. The possible role of these host protein candidates in CPMV infection was therefore further investigated (**Chapter 4**). Based on their predicted function, their known association with viral infection, and their abundance in the proteomic analysis three proteins were selected for further analysis: Heat shock protein (HSP) 60, an CDC48-type AAA-protein and a HSP70. These proteins are known to chaperone protein(complex) folding (HSP60), induce the recycling of misfolded MPs (CDC48) and are known to regulate both viral replication and virion translocation (HSP70). Through targeted gene silencing approaches the expression of these three proteins was knocked-down, and the effects of their altered expression on the viral infection cycle were examined. Silencing of either HSP70 or CDC48-protein isoforms resulted in a reduction of CPMV titres. Additionally, the silencing of these two proteins hampered the intercellular spread of CPMV and *Tobacco mosaic virus*. These results show that HSP70 and CDC48 are candidate components of CPMV induced movement complexes and also show that altering their expression influences the viral infection cycle. These results underscore the biological relevance of these proteins in CPMV infection.

The PD-located proteins (PDLPs) are found in PDs and are required for tubule-guided movement of *Grapevine fanleaf virus* (GFLV) and *Cauliflower mosaic virus* (CaMV). To test the requirement of these proteins in intercellular movement of CPMV the interaction between PDLPs and the CPMV MP was tested (**Chapter 5**). Using fluorescence lifetime imaging an interaction between this MP and a PDLP isoform inside the PD was established. The interaction of PDLP with the MP of CPMV was found at the same location and with similar binding kinetics as has been described for GFLV and CaMV, which suggests that the PD-located interactions with PDLPs could be a general requirement for the tubule-guided movement of viruses. To elucidate the relevance of the interaction between MPs and PDLPs localisation studies were conducted in protoplasts. The absence of PDs in these cells allows close examination of different stages of tubule assembly

to determine the significance of the observed MP-PLDP interactions. These experiments showed that PDLP1 did not co-localise with MPs in protoplasts, even though extensive co-localisation was observed in PDs. The observation that the MPs of CPMV and GFLV were able to form movement tubules on the surface of protoplasts without an interaction with PDLP, indicates that PDLP isoforms are not required for tubule initiation or for the anchoring of movement tubules to the plasma membrane in these cells. These results therefore imply that the interaction between tubule-forming MPs and PDLP is confined to the PD.

The results from this thesis have led to the proposition of a tentative model for the tubule-guided intercellular transport of CPMV between plant cells (**Chapter 6**). In this model the localised accumulation of MP at the plasma membrane leads to movement tubule assembly at the PD. Here HSP70s move the virion containing tubules into the modified PD channel, where PDLPs form a structure that guides the movement tubule through the PD channel. Intercellular transport is completed with the release of virus particles in the adjoining cells. This thesis research identified and characterized three host proteins that either interacted with (PDLP1) or influenced (HSP70/CDC48) the CPMV infection cycle, thereby paving the way for further studies to uncover the molecular mechanisms used by these proteins to accommodate CPMV intercellular spread.

Samenvatting

Verspreiding van plantenvirussen vanuit een geïnfecteerde cel naar de omliggende gezonde cellen vindt plaats via plasmodesmata (PDs). PDs zijn kanalen in de celwand tussen plantencellen waardoorheen het endoplasmatisch reticulum (ER), de celmembraan en het cytoplasma van naburige cellen met elkaar verbonden zijn en via welke de cellen met elkaar communiceren. Het intercellulaire transport van metabolieten en signaalstoffen, nodig voor de ontwikkeling van de plant, maar ook het intercellulaire transport van virale genomen of virusdeeltjes verloopt, via deze complexe poriën. Voor transport van het virale genoom of virusdeeltje moet de doorlaatbaarheid van deze PD worden aangepast (vergroot) en hierin spelen door het virus gecodeerde 'transporteiwitten' een belangrijke rol. Het *Cowpea mosaic virus* (CPMV, in het Nederlands: *Koebonenmozaïekvirus*) verspreidt zich als virusdeeltje via een 'transportbuis' die door het virus in de PD-porie wordt aangebracht. Deze buis is opgebouwd uit transporteiwitten. Om plaats te maken voor deze buis moet de structuur van het PD-kanaal worden aangepast. Zo moeten de PD-specifieke structuren zoals desmotubule (de verbinding van het ER tussen twee cellen) en structurele eiwitten worden verwijderd. Het tot stand komen van deze aanpassingen is zeer waarschijnlijk een gezamenlijke actie van componenten van zowel het virus als van de gastheer. Het doel van het hier beschreven onderzoek was dan ook om uit te zoeken welke gastheereiwitten nodig zijn voor het intercellulaire transport van plantenvirussen via transportbuizen en welke rol deze gastheereiwitten daarbij spelen.

Het plasmamembraan bekleedt de wanden van de PD en de assemblage van de transportbuis vindt daarom plaats in de nabijheid van dit membraan. De membraaneiwitten die zich in de plasmamembraan bevinden zouden een rol kunnen spelen bij bijv. de verankering van de buis. In een eerdere studie beschreef Carvalho (2003) een experiment waarin het transporteiwit van CPMV in vitro (in de reageerbuis) bindt aan een eiwit dat voorkomt in de plasmamembraan en behoort tot de groep van 'plasma membrane intrinsic proteins 1' (PIP1-eiwitten). In **Hoofdstuk 2** van dit proefschrift is de rol van PIP1-eiwitten in virustransport verder onderzocht. Het transporteiwit van CPMV en een PIP1-eiwit van *Nicotiana benthamiana* werden beide voorzien van een

fluorescerend label waarmee de lokalisatie en mogelijke co-lokalisatie van deze eiwitten kon worden onderzocht. Deze experimenten toonden aan dat *N. benthamiana* PIP1 en het transporteiwit van CPMV niet op dezelfde locatie in de cel aanwezig zijn en het dus onwaarschijnlijk is dat deze eiwitten een interactie met elkaar aangaan tijdens het infectieproces. Verder werden in *Nicotiana* protoplasten, waarin genen van meerdere PIP1-iso-vormen door middel van RNA interferentie stilgelegd waren, tóch transportbuizen gevormd. Dit was een extra aanwijzing dat buisvorming niet afhankelijk was van PIP1-activiteit.

Protoplasten zijn geïsoleerde plantencellen zonder celwand en dus zonder PDs. Na infectie van protoplasten met CPMV worden transportbuizen gevormd aan het celoppervlak. Deze buizen groeien uit in het medium en worden geheel omgeven door het plasmamembraan. Deze complexen van plasmamembraan en transportbuizen zijn gezuiverd door middel van immunoprecipitatie. Vervolgens werd door middel van tandem-massa-spectrometrie bepaald welke virus- en gastheereiwitten in de complexen aanwezig waren (**Hoofdstuk 3**). Deze proteoomanalyse resulteerde in de identificatie van een groot aantal gastheereiwitten, waarvan er zeven veelvoorkomend waren in het gezuiverde complex. Deze zeven eiwitten werden daarom geselecteerd als gastheercomponenten die mogelijk van belang zijn voor het cel-cel-transport en de samenstelling en positionering van de transportbuis. De rol van deze gastheereiwitten bij CPMV-infectie is daarom verder bestudeerd (**Hoofdstuk 4**). Drie van de zeven eiwitten, het 'heat shock protein' (HSP) 60, een CDC48 type AAA-eiwit en een HSP70, werden geselecteerd voor verder onderzoek op basis van reeds bekende betrokkenheid van deze eiwitten bij virale infecties, de mate van voorkomen in de proteoomanalyse en de aanwezigheid van specifieke eiwitdomeinen. Bekende functies van deze eiwitten zijn voor HSP60 het assisteren bij het vouwen van eiwitten (en eiwitcomplexen), voor CDC48 het recyclen van verkeerd gevouwen eiwitten en voor HSP70 o.a. een rol bij het transport en de replicatie van virussen. Door de genexpressie van deze eiwitten specifiek te belemmeren via RNA-interferentie (ook wel 'gene-silencing' genoemd) kunnen de effecten van verminderde genexpressie van gastheereiwitten op de virale infectie bestudeerd worden. De silencing van zowel HSP70, als CDC48-eiwitten in *N. benthamiana*-planten resulteerde in een significante afname van het aantal gevormde CPMV deeltjes. Daarnaast werd in

deze planten ook een verminderde verspreiding van zowel CPMV als het *Tobacco mosaic virus* geconstateerd. Deze resultaten laten zien dat HSP70 en CDC48 niet alleen mogelijke componenten zijn van het complex van plasmamembraan en transportbuis, maar dat deze ook van invloed zijn op de virale infectiecyclus en hierin mogelijk een biologisch relevante rol spelen.

In de PD zijn zogenaamde 'PD-located proteins' (PDLPs) aanwezig waarvan is aangetoond dat ze een interactie aangaan met de transporteiwitten van *Grapevine fanleaf virus* (GFLV) en *Cauliflouwer mosaic virus* (CaMV) en dat deze interactie vereist is voor het transport van virusdeeltjes via de transportbuizen. In **Hoofdstuk 5** is onderzocht of de interactie tussen deze PDLPs en het transporteiwit ook een vereiste is voor de verspreiding van CPMV. Met behulp van 'fluorescence lifetime imaging' kon een interactie worden vastgesteld tussen een PDLP-isovorm en het transporteiwit van CPMV in de PD. Wat betreft locatie en kinetiek was deze interactie gelijk aan die tussen PDLP en de transporteiwitten van GFLV en CaMV, wat doet vermoeden dat een dergelijke interactie een algemeen vereiste is voor het intercellulaire transport van buisvormende virussen. Om het mechanisme achter de interactie tussen transporteiwitten en PDLPs te achterhalen zijn lokalisatiestudies in protoplasten gedaan. Omdat deze geïsoleerde plantencellen geen PDs bevatten kunnen specifieke stadia in het proces van buisvorming in detail bestudeerd worden. Deze experimenten toonden aan dat PDLP1 niet co-lokaliseert met transporteiwitten in protoplasten, terwijl er wel een hoge mate van co-lokalisatie werd gevonden in PDs *in planta*. Deze observatie dat de transporteiwitten van zowel CPMV als GFLV in staat zijn om transportbuizen te vormen aan het oppervlak van protoplasten zonder een interactie aan te gaan met PDLP, duidt erop dat PDLP-eiwitten niet noodzakelijk zijn voor het initiëren van buisvorming of het verankeren van de transportbuis aan de plasmamembraan van deze cellen. Blijkbaar is een interactie tussen transporteiwitten en PDLPs alleen nodig voor vorming van de transportbuis in de PD.

De resultaten van dit proefschrift hebben geleid tot de formulering van een voorlopig model voor het intercellulaire transport van CPMV tussen plantencellen via transportbuizen in de PD (**Hoofdstuk 6**). In dit model verzamelen de transporteiwitten zich aan de plasmamembraan, wat leidt tot de vorming van transportbuizen in de PD. Hier faciliteert HSP70 de insertie van de transportbuis

mét virusdeeltjes in een structureel aangepaste PD-porie, waar een geleidende structuur van PDLP-eiwitten de transportbuis door het PD-kanaal leidt. Het intercellulaire transport wordt voltooid door het vrijkomen van de virusdeeltjes in de naburige cel.

Dit promotieonderzoek heeft geleid tot de identificatie en karakterisering van drie gastheereiwitten die ofwel interacteren (PDLP1) met ofwel invloed uitoefenen (HSP70/CDC48) op de CPMV-infectiecyclus. Hiermee is de basis gelegd voor gericht vervolgonderzoek naar de moleculaire mechanismen waarmee deze gastheereiwitten betrokken zijn bij het intercellulaire transport van CPMV.

About the author

Paul Wildrik den Hollander was born on the 26st of October 1983 in the Frisian town of Stavoren. In 2004 he started his biology study at the Wageningen University where he specialized in cellular plant biology. During his MSc studies he performed a master thesis at the Laboratory of Molecular biology studying the localization of methylation in arabidopsis nuclei. In a second master thesis he studied the weakening of the micropylar endosperm during tomato seed germination under the combined supervision of the Plant cell biology and Plant physiology labs of the Wageningen University. For his internship he visited the Wasteneys laboratory at the University of British Columbia in Vancouver, Canada, where he conducted research on the regulatory role of the cytoskeleton on arabidopsis root morphology. In 2009 he completed his MSc studies and joined the Laboratory of Virology of the Wageningen University as a PhD candidate. Here he studied the tubule-guided intercellular transport of plant viruses under the supervision of Jan van Lent, the results of this study are described in this thesis.

Currently, he is working as a lecturer in molecular plant biotechnology at the Inholland university of applied sciences in Amsterdam.

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life in Brazil! De lunchmeetings van de plantengroep waren altijd een goed klankbord, waar ik de input van Maarten, Martin en Myluska zeer op prijs heb gesteld. Ook de "TOSPO-boys" wil ik bedanken voor de discussies; Richard, voor de discussie over planten en virussen en ook voor de gezelligheid tijdens onze gezamenlijke meeting in mediterraan Frankrijk waar jij me aan menig planten viroloog introduceerde. Dryas, bedankt voor het doorsturen van de vacature, de luchtgitaar optredens in het lab en voor de botanische onderonsjes, zowel thuis als op het lab en zelfs in een kano op de Utrechtse grachten. Jij was de go-to persoon voor vele vragen en ik vond onze tijd op viro samen altijd gebroederlijk. Patrick, bedankt voor de hulp met alle bio-informatica, ik wens je alle goeds in Enkhuizen. Marcio thanks for the fun and the shenanigans in the lab (blot-football), good luck with the finalization of your thesis!
Daarnaast een bijzonder dankjewel voor mijn PhD lotgenoten, ik heb veel steun bij jullie gevonden. Het was heerlijk raaskallen bij jullie en ik voelde me door de gezamenlijke strijd met jullie verbonden. De borrels, de voetbalkampioenschappen op de WE-day (drie maal is scheepsrecht!), de verjaardagen en de carnaval kaassoufflés zijn goede herinnering (mag dat frituren in de fumehood?). Stefan bedankt voor je collegialiteit en het organiseren talloze activiteiten met als hoogtepunt de enige PHD trip ooit en wel naar de parel van het wad, Ameland! Veel geluk bij je avontuur in de nieuwe wereld! Stineke; wat hebben we het hosternokke gezellig gehad! Bedankt voor de koekjes bij de (goede) koffie, de gekkigheid en de zondagsschoolliedjes. Maar ook buiten het lab, de gastvrijheid op de Buissteeg als ik weer eens uilen wilde kijken of een bordje eten nodig had. Ik kom zo snel mogelijk in Cornwall naar de bosuilen, dassen, vossen en groentes in de nieuwe tuin kijken! Peng Ke, my Chinese friend, I enjoyed the fun time we had as roommates when I stared my PhD. You showed me the best sites in Heidelberg and introduced me to real Chinese food, I wish you well! Jelke het was altijd leuk kletsen in de snelste Peugeot van Utrecht, succes met het publiceren van al je hoofdstukken. Athos, my worst friend, I was ending my PhD when you were starting yours. I enjoyed (y)our type of humor, the movies we talked about and the discussions on various scientific subjects. I am glad to see your still bringing sandwiches to lunch in that red and blue box and look forward to seeing you solve the Sw5 puzzle! Mia voor de gezelligheid en de vrolijkheid die je in het lab brengt, succes met het immuniseren van de Noorse

zalmen. Amaya, you beat me to it, your defence is before mine! The race is over and I congratulate you ;) !
Wie ik ook zeker moet bedanken is Corrinne, waar zou het virologie lab zonder jou zijn? Het lijkt soms of je alles voor ons in goede banen leidt, zowel in het lab, als bij activiteiten als de vrijdagmiddagborrel. Ik ben je veel dank verschuldigd voor het eindeloos naar de promega-vriezer lopen, voor alle spullen die ik van je heb geleend en omdat ik vaak "stiekem" na boodschappentijd toch nog mijn vergeten spulletjes in mijn ijsbak vond. Maar naast een fijne collega ben je ook nog: altijd op tijd met het ophangen van verjaardagslingers en een geweldige carpoolchauffeur, zonder jou was ik er letterlijk niet gekomen. Ik vind het echt tof dat je, hoogzwanger en wel, mijn paranimf wilt zijn.
Ook de overige leden van virologie die direct of indirect een bijdrage hebben geleverd aan dit proefschrift wil ik graag bedankten. Onze "nieuwe professor" Monique, voor de interesse in mijn project en de motiverende woorden. Gorben voor de kritische vragen bij de maandag morgen presentaties. Vera, voor de hulp bij de statistiek en natuurlijk voor de ontelbare ritjes van en naar Wageningen in de Volvo. Tot ziens in Utrecht of op de spinfiets! Ook wil ik Dick Lohuis bedanken voor alle hulp in het lab en voor de vriendelijke praatjes tijdens wachtstappen. Er was geen bandje wat jij niet kon verklaren, geen vreemde blot waar je geen verklaring voor wist, je voorraad buffers en je vele "truckjes" maakten het labwerk sneller makkelijker en leuker. Ook wil ik Janneke en Dick Peters bedanken voor al het advies in de kassen waar ik vaak veel aan had. Marleen, jou wil ik bedanken voor het regelen van zo'n beetje alles, EBS nummers, vliegtickets, zoek geraakte orders, studentenverslagen, als ik jou om hulp vroeg kwam het altijd goed!
Ik heb met veel genoegen een handje vol studenten begeleid in thesis projecten, ik wil hen allen bedanken! Het is jammer dat hun harde werk niet altijd in dit boekje terecht is gekomen. Anne, bedankt voor je hulp bij het maken van het TC-gelabelde virus. Beata, thank you for your efforts spend on silencing of the PIP genes. Saskia, bedankt voor het eindeloos speuren naar punctate peripheral spots. En Sebastian bedankt voor de toffe samenwerking aan de PDLP eiwitten. Misschien is het wel doordat jullie van die voorbeeldige studenten waren, dat ik als docent plantenbiotechnologie begonnen ben aan de Hogeschool Inholland. Ik wil hier ook graag mijn nieuwe collega's uit Amsterdam bedanken voor de

interesse en morele steun bij de laatste loodjes van mijn PhD traject.
Ook wil ik de mensen bedanken die mij hebben voorzien van technische ondersteuning bij het gebruik van allerhande gecompliceerde machines. Onder andere Sjef en Jan-Willem die mij op het Biotechnion de weg wezen en uitstekend hebben begeleid bij de MS/MS analyse en FLIM metingen. Op de campus waren Norbert en Henk Schipper er altijd als een confocale microscoop niet deed wat hij moest doen, bedankt voor de technische ondersteuning. Hier noem ik ook de vakgroep moleculaire biologie waar ik altijd terecht kon voor advies, protocollen, microscopen, plasmides of chemicaliën die bij ons op waren, Bedankt! Ook wil ik de medewerkers van Unifarm bedanken voor het verzorgen en opgroeien van mijn planten. Ik ben vooral Henk Smid dank verschuldigd voor het (ook op zeer korte termijn) regelen van de benodigde planten, je geïnteresseerde vragen maakten de uren in de soms erg warme kas aangenamer.

Maar een PhD doe je niet allen in de werkweek, PhD'er ben je 24-7, daarom wil ik ook mijn familie bedanken. Zowel mijn eigen Friese gezinsleden als mijn schoonfamilie. Voor jullie interesse, de opbeurende woorden of gewoon het luisteren naar mijn (onbegrijpelijke) verhalen over het transport van planten virussen. "De Krakers" Thijs, Pascal, Lenny, Rik, Kees jullie wil ik bedanken voor de uurtjes stoom afblazen op maandag, de interessante gesprekken, en de ongelofelijke hoeveelheid onzin waar jullie mij mee wisten te vermaken. Maar ik ben ook erg bij dat we ook over serieuze zaken kunnen praten, dat hielp mij vaak weer op weg. De gedeelde concertjes, de gedronken biertjes, de gefietste kilometers en de stellingen (er gebeurde nogal wat op de LA166). Maar natuurlijk was het muziek maken het eigenlijke doel, als ik op maandag niet had kunnen "blèren" weet ik niet of het met mijn PhD project goed was afgelopen. Dat we maar de hoogste opgeleide "band" van Nederland mogen worden Lenny, Rik, Kees wie schrijft de volgende thesis?
Een ander goed moment om de gedachten op een rij te zetten waren de autoritjes naar de uithoeken van het land waar de zeldzame vogels zich ophielden. Joost, de vogels waren een excuus voor een gezamenlijke activiteit, nu zijn de vogels voor bijna alles een excuus. Ik geniet van de dagen dat we in de buitenlucht speuren naar al wat voorbij vliegt, dat het promotie traject is afgerond betekend zeker niet dat we niets meer te kletsen hebben in de

auto, zolang er vogels vliegen blijven we gaan zeg ik! Het hoogtepunt van onze vogelgekte: de spaanse vogel-excursie, 16 uur per dag door de Pyreneeën scheuren, vogels kijken en vergeten dat je ook nog had willen lunchen! Ik ben erg blij dat we daar vergezeld werden door Edze en niet alleen vanwege de verstopte eieren. Edze, de wetenschappelijke onderonsjes die naar mij idee op de achterbank van de amazing Mazda begonnen en voortgezet werden op de buissteeg en in de uiterwaarden, met altijd als excuus vogels kijken, hebben mij geholpen een kritischere wetenschapper te worden. Ik hoop dat er in de toekomst nog vele vogelexcursies zullen volgen.
Ook wil ik de vrienden uit "ût hokje" bedanken voor de afleiding de afgelopen jaren. Arjen, met jou kon ik uren fietsen, onze gesprekken tijdens deze tochtjes hebben zeker geholpen om het hoofd leeg te trappen, wat mij altijd vooruit geholpen heeft. Laten we deze sportieve traditie hoog houden en 240 km door "it heitelân" opnemen in de sportieve kalender! Jan, bedankt voor de avonden vermaak met zombies en super Mario! Tseard en (andere) Jan bedankt voor de mooie dagen headbangen!
En dan, last but certainly not least, Karlijn, jou ben ik natuurlijk super dankbaar. Jij bent er altijd voor mij. Als ik een baaldag had was jij een luisterend oor en hielp je mij te relativeren, op een goede dag wachtte je me thuis op met taart, met jou voel ik me nooit alleen. Bedankt dat je mij op de rails hebt gehouden deze vijf jaar ook al moest je daar soms jouw dingen voor aan de kant zetten, je bent met recht Frau Doktor. Al 11 jaar gaan we samen door het leven, vinden we steun bij elkaar en bezoeken we de mooiste plekken van de wereld. Ik hoop dat dat voor altijd zo blijft.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Paulus W. den Hollander
Date: 18 November 2014
Group: Virology
University: Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Plasmodesmal dynamics related to virus movement	Jan 18, 2010
► **Writing or rewriting a project proposal**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Course 'Safe handling with radioactive materials and sources', expert 5B	May 25-29, 2010
Subtotal Start-up Phase	*3.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Students Day, Utrecht University	Jun 01, 2010
EPS PhD Students Day, Wageningen University	May 20, 2011
ExPectationS day (EPS Career Day)	Feb 01, 2013
► **EPS theme symposia**	
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 03, 2011
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Wageningen University	Feb 10, 2012
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 24, 2013
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 19-20, 2010
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 04-05, 2011
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 02-03, 2012
NWO-ALW meeting Exp. Plant Sciences, Lunteren	Apr 22-23, 2013
Annual meeting "Nedelandse vereniging voor microscopie", Veldhoven, NL	Oct 01-02, 2012
► **Seminars (series), workshops and symposia**	
Plant science seminars 'Mathematics and statistical methods' and 'Phytopathology'	Nov 10, 2009
Invited seminar by SF Elena, 'Mechanisms of genetic robustness in RNA viruses'	Nov 19, 2009
Plant science seminars 'Entomology' and 'Plant Cell Biology''	Jan 12,2010
Invited seminar by Peter Moffett, 'Constitutive and R gene-induced defences against plant viruses'	Feb 05,2010
Dutch Annual Virology Symposium	Mar 05, 2010
EPS workshop "Plant Endomembranes", VU, Amsterdam	Jul 02, 2010
Invited seminar by Chris Hawes, 'Shaping the plant endomembrane system'	Aug 20, 2010
PhD lecture 'writing a world class paper'	Oct 26, 2010
Dutch Annual Virology Symposium	Mar 18, 2011
Dutch Annual Virology Symposium	Mar 02, 2012
WEES seminar by Michael Strand, 'Viruses as beneficial symbionts of insects: the evolution and function of the Polydnaviridae'	Apr 26, 2012
Invited seminar by Patrick Forterre, 'New concepts on the origin and nature of viruses: their major role in both ancient and recent biological evolution'.	Oct 18, 2012
Mini symposium 'Frontiers in plant morphogenesis'	Nov 13, 2012
Science webinar, 'Microscopy in Focus: The Art and Science of Image Quality'	Mar 07, 2013
Invited seminar by Rüdiger Simon, 'Plant stem cell systems'	Apr 15, 2013
Invited seminar by Jolanda Smit, 'Viruses transmitted by mosquitos'	May 21, 2013
Invited seminar by Stephane Blanc, 'New insights into the relationship between plant viruses and insect vectors'	Sep 18, 2013
► **Seminar plus**	
Master class by Michael Strand, 'Viruses as beneficial symbionts of insects'	Apr 26, 2011
► **International symposia and congresses**	
Botanical microscopy meeting, Wageingen University, NL	Apr 16-21, 2011
EMBO meeting 'Green viruses, from gene to landscape', Hyères-les-Palmiers, France	Sep 07-11, 2013
► **Presentations**	
Poster: 'Structures and proteins involved in cowpea mosaic virus cell-to-cell transport', Spring School, Wageningen	Apr 15, 2010
Poster: 'Identification of host proteins involved in cell-to-cell movement of Cowpea Mosaic Virus', Autumn School, Wageningen	Nov 02, 2011
Oral: 'Identification and characterization of host proteins involved in the formation of Cowpea mosaic virus movement tubules in the plasma membrane', EPS Theme 2, Wageningen	Feb 10, 2012

CONTINUED ON NEXT PAGE

Poster: 'Tomographic analysis of (CPMV) intercellular movement tubules', Veldhoven, NL	Oct 02, 2012
Poster: 'Proteomic and genetic analysis of host protein involvement in CPMV intercellular movement', ALW meeting, Lunteren, NL	Apr 22, 2013
Oral: 'Proteomic analysis of the plasma membrane / movement tubule complex of Cowpea mosaic virus', EMBO meeting, France	Sep 08, 2013
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Nov 14, 2012
► **Excursions**	
Subtotal Scientific Exposure	*17.3 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
ESP Spring School 'RNAi and the world of small RNA molecules'	Apr 14-16, 2010
EPS PhD course 'An introduction to electron microscopy'	Jun 21-25, 2010
EPS Autumn School 'Host-Microbe Interactomics'	Nov 01-03, 2011
EPS PhD course 'Bioinformatics A users approach'	Aug 27-31, 2012
EMBO practical course 'Electron Microscopy and Stereology in Cell Biology'	Jun 16-26, 2013
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	*credits**

4) Personal development	*date*
► **Skill training courses**	
PhD competence assessment	Jan 19, 2010
Course 'Project- and time management'	2011
Course 'Scientific writing'	Jan-Feb 2013
Course 'Techniques for Writing and Presenting a Scientific Paper'	Feb 05-08, 2013
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.2 credits**

TOTAL NUMBER OF CREDIT POINTS*	**32,3**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	
** A credit represents a normative study load of 28 hours of study.*	

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