Mechanisms underlying

Cowpea mosaic virus systemic infection

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I dedicate this thesis to God, my Creator and Saviour,
in His Honour and for His Glorification,
and to my beloved parents, whose 'yes' allowed
the Lord to give me the gift of life.

Magnificat

And she said:
"My soul proclaims the greatness of the Lord
and my spirit exults in God my Saviour,
because he has looked upon his lowly handmaid.
Yes! From this day forward
all generations will call me blessed,
for the Almighty has done great things for me.
Holy is his name
and mercy reaches from age to age
for those who fear him.
He has shown the power of his arm,
he has routed the proud of heart.
He has pulled down princes from their thrones
and exalted the lowly.
The hungry he has filled with good things,
the rich sent empty away.
He has come to the help of Israel his servant,
mindful of his mercy,
according to the promise he made to our ancestors,
of his mercy to Abraham and to his descendant,
forever!" (Luke 1, 46-55)

Magnificat

Então ela disse:
"Minha alma engrandece o Senhor,
e meu espírito exulta em Deus meu Salvador,
porque olhou para a humilhação de sua serva.
Sim! Doravante as gerações todas
me chamarão de bem-aventurada, pois
o Todo-podereoso fez grandes coisas em meu favor.
Seu nome é Santo
e sua misericórdia perdura de geração em geração,
para aqueles que o temem.
Agiu com a força de seu braço.
Dispersou os homens de coração orgulhoso.
Depôs poderosos de seus tronos,
e a humildes exaltou.
Cumulou de bens os famintos
e despediu ricos de mãos vazias.
Socorreu Israel, seu servo,
lembrando de sua misericórdia,
conforme prometera a nossos pais,
em favor de Abraão e de sua descendência,
para sempre!" (Lucas 1, 46-55)

About the cover: Veinal network of a cowpea leaf visualised by the uptake of red fluorescing Texas Red dextran; artificially coloured blue.
For details see Chapter 2 of this dissertation.

Contents

CHAPTER 1

General Introduction

Systemic infection of plants by pathogenic viruses involves (i) (vector-mediated) inoculation of the virus, (ii) replication in the primarily inoculated cell, (iii) local cell-to-cell spread within the inoculated plant organ (e.g. leaf, stem, roots) and (iv) systemic spread through the plant via the vascular system. Furthermore, the success of virus infection in plants is the consequence of competition between virus replication/spread and plant anti-viral defence mechanisms. Plants have developed such defence mechanisms to arrest viral infection by, for instance, encoding dominant resistance genes that trigger a hypersensitive response (HR) or by sequence-specific degradation of viral RNA through a mechanism known as RNA silencing (reviewed by Vance & Vaucheret, 2001; Goldbach *et al.*, 2003), among other strategies.

In comparison to animal viruses, plant-infecting viruses encounter two extra barriers to invade their host cells: the cuticle and the cell wall. For that reason, plant viruses can only enter their host through a transiently wounded cell (reviewed by Verduin, 1992). Consequently, horizontal transmission of plant viruses must be mediated either by biological vectors such as insects, fungi and nematodes (reviewed by Campbell, 1996; Power, 2000; Brown & MacFarlane, 2001; Rush, 2003) or by mechanical inoculation with abrasives. Once in the cytoplasm, the virion must be disassembled to make the viral genome available for the translation and replication machineries (reviewed by Maia & Haenni, 1994). Replication requires the viral encoded polymerase in conjunction with host factors (reviewed by Hull, 2001).

Viruses spread from the initially infected cells into adjacent ones through complex cytoplasmic connections denoted plasmodesmata (PD), a process which is generally referred to as cell-to-cell movement (reviewed by Carrington *et al.*, 1996; Roberts & Oparka, 2003). However, PD are tightly regulated channels that in normal state only allow passage of molecules smaller than 1 kDa, thus free passage of the much larger virions or viral nucleoprotein complexes is excluded (Terry & Robards, 1987; Wolf *et al.*, 1989). To achieve movement of such viral complexes, plant viruses encode so-called movement proteins (MPs), whose function is to modify and gate the PD (reviewed by Lazarowitz & Beachy, 1999). Two main strategies for cell-to-cell movement have been described so far: the first, exemplified by the tobamoviruses, concerns cell-to-cell movement of a viral RNA-MP complex in a non virion form (reviewed by Rhee *et al.*, 2000); the second is based on tubule-guided movement of mature virions or non-enveloped nucleocapsids through tubules built-up from the MPs, e.g. como- (van Lent *et al.*, 1990), nepo- (Wieczorek & Sanfaçon, 1993; Ritzenthaler

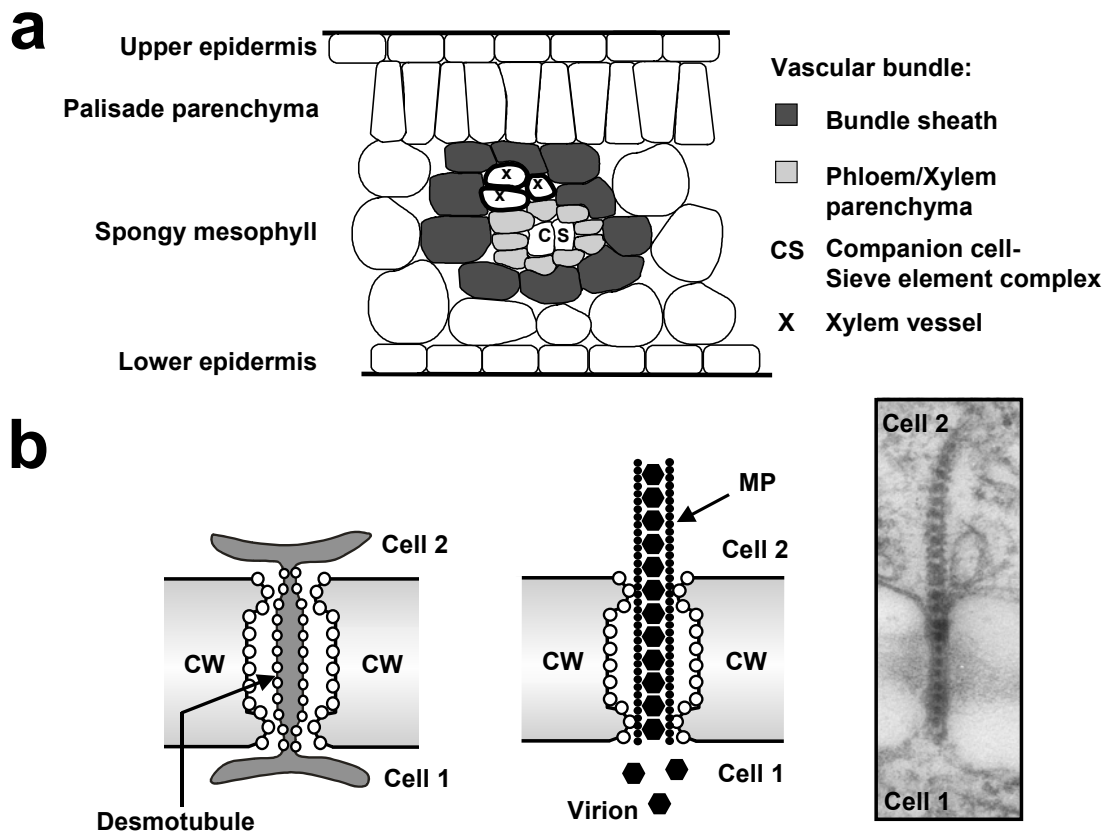

Figure 1. (a) Schematic representation of cross section through the leaf lamina displays various cell types which can be infected by viruses. (b) Schematic representation of a mesophyll plasmodesma connecting adjacent cells (left). Desmotubule is the portion of endoplasmic reticulum that passes through the plasmodesmal pore. CW, cell wall. Open circles represent plasmodesmal proteins. Schematic representation (middle) and micrograph (right) showing plasmodesmata modified by *Cowpea mosaic virus* (CPMV) for tubule-guided cell-to-cell movement of virions. The tubule is build-up from units of CPMV encoded movement protein (MP).

et al., 1995), caulimo- (Kitajima & Lauritis, 1969; Kasteel *et al.*, 1996; Huang *et al.*, 2001), badna- (Cheng *et al.*, 1998), bromo- (Kasteel *et al.*, 1997; van der Wel *et al.*, 2000) and tospoviruses (Storms *et al.*, 1995).

Virus replication and local spread occurs in various cell types within the inoculated leaf until the vascular bundle is reached, from where the virus spreads systemically (Fig. 1a). The bundle sheath-phloem parenchyma (BS-PP) boundary represents the barrier that separates local cell-to-cell movement and systemic spread (Ding *et al.*, 1992, Lucas & Gilbertson, 1994; Thompson & García-Arenal, 1998). Three stages of the systemic spread, also denoted long-distance or vascular movement, can be distinguished: entry into (loading), translocation through and exit from (unloading) the phloem (Séron & Haenni, 1996). Virus is loaded into and unloaded from the sieve elements (SE) through SE-specific plasmodesmata named pore-plasmodesma-units (PPU) (reviewed by van Bel & Kempers, 1997). The PPU possess a unique morphology consisting of a single pore on the SE side that extensively

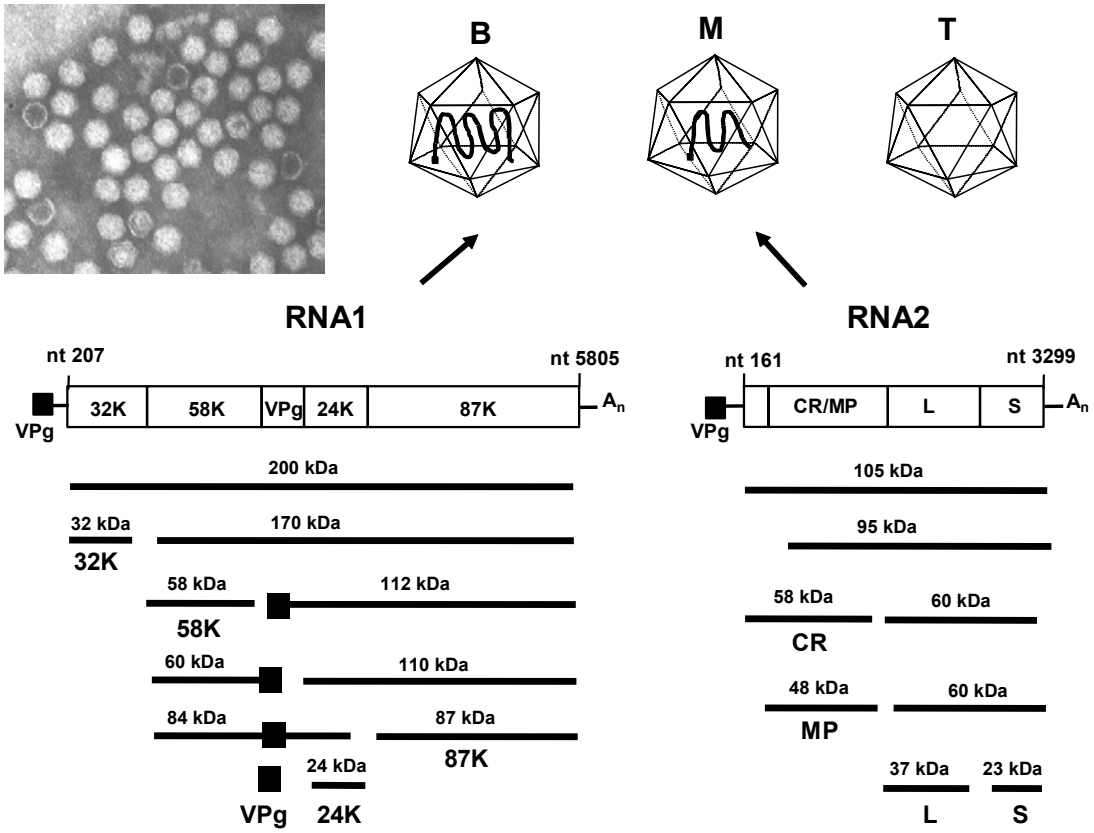

Figure 2. *Cowpea mosaic virus* (CPMV) genomic organization. The genome is bipartite in segments of single stranded RNAs denoted RNA1 and RNA2, which are encapsidated in separate spherical virions, the bottom component (B) and middle component (M). The top component (T) comprises empty virions devoid of genetic material. The micrograph on the left shows purified CPMV virions of about 29 nm in diameter. Both RNA segments contain a small viral protein (VPg) at their 5' end and a poly-A tail (A_n) at their 3' end. The translatable region is comprised between the nucleotides (nt) 207-5805 for RNA1 and nt 161 and 3299 for RNA2. The RNA1 encodes the proteins involved in viral replication, namely the RNA-dependent RNA-polymerase (87K), the protease (24K), the cofactor of the protease (32K), the VPg and the 60 kDa putative helicase (58K+VPg). The RNA2 encodes a cofactor for RNA2 replication (CR) and the proteins involved in virus cell-to-cell movement, namely the movement protein (MP), the large coat protein (L) and the small coat protein (S).

branches into the neighbouring companion cell (CC) side (reviewed by Leisner & Turgeon, 1993). Besides being morphologically different from the PD in mesophyll cells, the PPU are also functionally different in that they usually have higher size exclusion limits (SEL), allowing passage of large molecules (Kempers *et al.,* 1993; van Bel, 1996; Kempers & van Bel, 1997; Turgeon, 2000; Fisher & Cash-Clark, 2000). Due to such anatomical and physiological particularities inherent to the mesophyll and vascular tissues, the vascular movement of viruses involves viral and host functions that are distinct from those associated with cell-to-cell movement (reviewed by Oparka & Santa-Cruz, 2000).

The transmission, replication and cell-to-cell movement of plant viruses have been well studied for several vector-virus-plant systems, but in general the vascular movement mechanism and the host determinants required for the systemic infection

process are much less understood. In this thesis research, studies were concentrated on the vascular movement mechanisms of *Cowpea mosaic virus* and on the barriers imposed by various plant hosts against systemic infection by this virus.

Cowpea mosaic virus

Cowpea mosaic virus (CPMV) is the type member of the *Comoviridae* family and has a bipartite genome, i.e. its genetic information being divided over two positive-sense single stranded RNA segments (RNA1 and RNA2), each encapsidated in separate spherical virions of about 29 nm in diameter (Fig. 2; reviewed by Goldbach & Wellink, 1996). The expression strategy is based on the production of large polyproteins which are proteolytically cleaved into 16 intermediate and final products by the virally encoded 24 kDa proteinase (24K) (Fig. 2; reviewed by Pouwels *et al.*, 2002a). Both RNA segments contain a small viral protein (VPg) at their 5' end and a poly-A tail at their 3' end. The proteins involved in replication are encoded by RNA1: the 32 kDa protein (32K) is involved in regulation of RNA1 polyprotein processing and required as cofactor for cleavage of the RNA2 polyprotein (Peters *et al.*, 1992), the 60 kDa protein (58K + VPg) is a putative viral helicase (Peters *et al.*, 1994), the VPg a putative primer for RNA transcription, the 87 kDa protein (87K) is the RNA-dependent RNA polymerase (RdRp) and the 24 kDa protein (24K) is the protease. Although the 87K has a specific domain for RdRp, the 110K fusion (87K + 24K) is probably required for the 87K RNA polymerase activity (Dorssers *et al.*, 1984). CPMV RNA replication is closely associated with membranous vesicles originating from the endoplasmic reticulum (ER) (de Zoeten *et al.*, 1974; Carette *et al.*, 2000; 2002a). The RNA2 encodes for the 58 kDa cofactor (CR) required for replication of RNA2 and proteins involved in cell-to-cell movement, i.e. the 48 kDa movement protein (MP), the 37 kDa large (L) and 23 kDa small (S) coat proteins.

Cell-to-cell movement of CPMV occurs through tubular structures, built-up from the viral MP, that replace the desmotubule (ER portion inside the PD) and through which mature virions are transported from one cell into the adjacent ones (Fig. 1b; Wellink & van Kammen, 1989; van Lent *et al.*, 1990). Tubular structures filled with virions, similar to those found in plant tissue, are also formed in CPMV-infected protoplasts (van Lent *et al.*, 1991). It was demonstrated by mutational analysis that the MP is the only viral protein required for tubule formation (Kasteel *et al.*, 1993; Welllink *et al.*, 1993). The ability of the MP to form tubular structures in insect cells (Kasteel *et al.*, 1996) indicates that PD or their remnants are not essential for initiation of tubule formation, and if host proteins are involved in the process they must be conserved among plants and animals. Studies with metabolic inhibitors indicate that the targeting of the MP to the periphery of the cell does not involve the cytoskeleton or

the secretory pathway, suggesting that the MP simply arrives at the plasma membrane by diffusion (Pouwels *et al.*, 2002b). Either dimerisation or multimerisation of the MP has been shown to be a requisite for its accumulation in the periphery of the cell (Pouwels *et al.*, 2003). It has been demonstrated that the CPMV MP binds exclusively to its homologous virions and that the L coat protein is involved in this binding (Carvalho *et al.*, 2003). Moreover, the MP binds rGTP but no other rNTPs, and a GTP-binding site was located within the sequence motif conserved among the MPs of tobamoviruses and comoviruses (Carvalho *et al.*, 2004). Although the CPMV MP is capable of binding single stranded RNA and DNA *in vitro*, but not double stranded nucleic acids, in a sequence non-specific manner (Carvalho *et al.*, 2004), the biological significance of this property still needs to be established. Considering the various observations available to date, a speculative model for the cell-to-cell movement of CPMV has been recently proposed (Pouwels *et al.*, 2002a; Carvalho, 2003): The MP, possibly in dimeric or multimeric form, binds to the L coat protein at the virus replication/assembly site and the complex is targeted to the plasma membrane by a mechanism that does not involve the cytoskeleton or ER membranes. The MP may then anchor to plasma membrane-residing proteins and polymerises within the PD pore into a tubules, thereby encaging the virions. GTP hydrolysis would take place to provide energy for tubule-assembly and filling of tubules with virions. At the same time, hydrolysis of the MP-bound GTP would destabilise the tubule in the neighbouring uninfected cell and result in tubule disintegration to release the virions for further infection.

In contrary to the cell-to-cell movement, the mechanism of vascular movement of CPMV is still enigmatic and interesting questions are whether it is also tubule-guided, like cell-to-cell movement, whether virions or other type of ribonucleoprotein complexes are transported through the vasculature and which viral and host factors participate in this process.

Viral factors involved in vascular movement of plant viruses

For several plant virus-host combinations, there are evidences for circulation of either virions or other types of ribonucleoprotein complexes along the vascular stream, with both structural and non-structural viral proteins playing a role in the various vascular movement mechanisms (reviewed by Santa-Cruz, 1999; Thompson & Schulz, 1999; Oparka & Santa-Cruz, 2000). In general a distinction can be made between viruses that require and those that do not require the coat protein (CP) for vascular movement (reviewed by Séron & Haenni, 1996). Following this distinction, in the next two sections an overview is given of the vascular movement of viruses from different virus genera.

CP-dependent vascular movement

Tobamoviruses - *Tobacco mosaic virus* (TMV) requires the CP for efficient systemic spread in tobacco plants (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Ding *et al*., 1996), although for local spread the CP is dispensable (reviewed by Carrington *et al.*, 1996). It has been suggested that the CPs must assemble into virions to enable vascular movement of TMV virus particles in the phloem (Esau & Cronshaw, 1967; Saito *et al.*, 1990). However, there is some evidence that other types of non-virion RNA-CP complexes may also be systemically transported in tobacco (Dorokhov *et al*., 1983; 1984). Also the role of TMV MP in vascular movement is controversial (Gera *et al.*, 1995; Arce-Johnson *et al.*, 1997). The masked strain of TMV (M-TMV), which encodes a mutant replicase, has its vascular movement impeded at the level of phloem loading, although its ability to replicate is not disturbed (Holt *et al*., 1990; Nelson *et al.*, 1993; Ding, X.S. *et al.*, 1995). This provides indirect evidence for the involvement of the viral replicase gene or protein in TMV vascular movement.

Potyviruses - The vascular movement of a *Tobacco etch virus* (TEV) mutant defective in the CP can be rescued by a transgenic plant expressing the CP (Dolja *et al.*, 1995). Hence, as for cell-to-cell movement (Rodriguez-Cerezo *et al.*, 1997; Rojas *et al.*, 1997), TEV requires the CP (Dolja *et al.*, 1995) for systemic spread. Furthermore, a mutation in the TEV CP inhibits genome encapsidation but does not abolish the systemic infection (Dolja *et al.*, 1994), demonstrating that virion formation is not essential for spread of this virus. There is also evidence that the VPg, which is covalently attached to the 5' end of the viral genomic RNA, interacts either directly or indirectly with host components to facilitate the TEV vascular movement (Schaad *et al.*, 1997). Likewise, the hindrance of vascular movement of *Potato virus A* (PVA) isolate M (PVA-M) in the solanaceae *Nicandra physaloides* is complemented by the 6K2 protein and VPg of the isolate B11 (PVA-B11), suggesting an involvement of these two proteins in potyviral movement through the phloem (Rajamäki & Valkonen, 1999). The VPg controls the phloem loading of PVA in potato as well (Rajamäki & Valkonen, 2002), and seems to be translocated from inoculated source to non-inoculated sink leaves, where it accumulates in CC at early stages of the infection, probably facilitating phloem unloading of the virus (Rajamäki & Valkonen, 2003).

A TEV mutant in the helper-component proteinase (HC-Pro) is able to move from cell-to-cell and reach the SE, but is impaired in further vascular movement (Cronin *et al*., 1995). HC-Pro is a multifunctional protein involved in aphid transmission of virions, auto-proteolytic processing, viral RNA replication, virus local and systemic spread (reviewed by: Maia *et al.*, 1996; Urcuqui-Inchima *et al.*, 2001; Plisson *et al.*, 2003) and suppression of RNA silencing (Anandalakshmi *et al.*, 1998; Kasschau &

Carrington, 1998). HC-Pro may directly interact with the virus to transport it through the PPU and/or indirectly favour phloem (un)loading by combating RNA silencing within the vasculature. Interestingly, there are data suggesting that RNA silencing could be more efficient within the phloem tissue than in mesophyll cells (Balmori-Melian *et al.*, 2002). Cronin *et al.* (1995) proposed that vascular movement of TEV involves an interaction between HC-Pro and CP (or virions). Roudet-Tavert *et al*. (2002) demonstrated interaction *in planta* between *Lettuce mosaic potyvirus* (LMV) HC-Pro and three different potyviral CPs and that the interaction was not abolished when a potyviral CP with a mutation in the domain involved in aphid-transmission of virions was used. Furthermore, HC-Pro was not detected in immunosorbent electron microscopy of virions present in infected leaf extracts (Roudet-Tavert *et al*., 2002), in spite the fact that HC-Pro necessarily interacts with virions in aphids for vector transmission (Thornbury *et al*., 1985; Wang, R. Y. *et al.*, 1996). Thus, it was suggested that HC-Pro may interact with the CP in a non-virion form possibly during the vascular movement process. Studies with TEV variants containing mutations in HC-Pro indicate that the role of HC-Pro in vascular movement and genome replication depends on its RNA silencing suppression activity and this is apparently independent of HC-Pro proteolytic activity (Kasschau & Carrington, 2001). Considering all the evidences together, transport of potyviral RNA through PPU probably occurs in non-virion form, i.e. as ribonucleoprotein complex (viral RNA, VPg, CP and HC-Pro), and this process is possibly facilitated by HC-Pro interaction with the CP and regulated by VPg.

Cucumoviruses - In the case of *Cucumber mosaic virus* (CMV), which needs the CP for cell-to-cell movement (Suzuki *et al.*, 1991; Canto *et al.*, 1997), a discrete mutation in the N-terminal region of the CP still permits the cell-to-cell movement of the virus but it abolishes vascular movement (Suzuki *et al*., 1991). CMV virions have been detected in vesicle aggregates in mature SEs (Blackman *et al*., 1998). For CMV, it was postulated that before the virus is loaded into the SE, virus particles disassemble in the cytoplasm of CC, move through the PPU as a ribonucleoprotein complex and reassemble in the SE (Blackman *et al*., 1998). The CMV 3a MP has been shown to move through PPUs (Itaya *et al.*, 2002), but the biological relevance of this observation for CMV systemic movement is unclear. The CMV 2b protein was identified as co-factor facilitating virus systemic movement (Ding, S.W. *et al.*, 1995), however, more recently 2b has been shown to suppress a systemic signalling step in the RNA silencing pathway (Brigneti *et al.*, 1998). Apparently, the role of 2b in CMV systemic spread is to counteract a plant resistance mechanism against systemic infection rather than directly promote the virus phloem (un)loading.

Potexviruses - Despite the absolute requirement of the CP for cell-to-cell movement (Chapman *et al.*, 1992a, 1992b; Baulcombe *et al.*, 1995), it is not

completely certain whether the CP is equally indispensable for vascular movement of potexviruses (Santa-Cruz *et al*., 1998). Nevertheless, by using a *Potato virus X* (PVX) CP mutant in a *trans*-complementation and a graft system, it was demonstrated that the CP has the ability to enter, translocate and exit from the phloem (Santa-Cruz *et al*., 1998). The CP of PVX localizes to mesophyll PD until the bundle sheath boundary, but is not detected in PD connecting phloem parenchyma to CC or in the PPU (Santa-Cruz *et al*., 1998), what could indicate that PVX is loaded into the phloem in a non-virion form. The vascular movement of *White clover mosaic virus* (WClMV) apparently involves the transport of a ribonucleoprotein complex containing the viral RNA, CP and the triple gene block (TGB) p1 MP (Lough *et al.*, 2001). Once loaded into the phloem stream, this complex can exit in sink tissues and replicate in the absence of the TGB p2/p3 MPs (Lough *et al.*, 2001), despite the requirement of all three TGB MPs for cell-to-cell movement (reviewed by Morozov & Solovyev, 2003). Voinnet *et al.* (2000) demonstrated that PVX TGB p1 is a suppressor of RNA silencing. Therefore, TGB p1 present in the phloem may interact with viral/host factors required for viral phloem (un)loading and/or may act as a silencing suppressor within the vascular tissue.

Closteroviruses - The p20 protein of *Beet yellows virus* (BYV), dispensable for BYV cell-to-cell movement, was found to be essential for virus vascular movement and to interact with Hsp70 homolog (Hsp70h) MP bound to virions (Prokhnevsky *et al.*, 2002). Either p20 associates with virions and Hsp70h mediates the transport of BYV through PPU, or p20 stabilises the virion within the alkaline environment of the phloem sap, or even protects virions from inactivation by plant defence factors present in the phloem (Prokhnevsky *et al.*, 2002). Furthermore, the BYV leader proteinase (L-Pro), which is also a replication enhancer, is not required for cell-to-cell movement but is necessary for viral vascular movement (Peng *et al.*, 2003). L-Pro has no RNA silencing suppression activity, which recently was shown to be a function of the protein p21 (Reed *et al.*, 2003). Thus, it can be that L-Pro promotes BYV replication specifically within the phloem and/or that it interferes with host defence responses in this tissue (Peng *et al.*, 2003). It must be emphasized that the movement mechanisms employed by BYV may be unique among viruses of the *Closteroviridae* family, since BYV is the only known closterovirus capable of phloem unloading (Prokhnevsky *et al.*, 2002).

CP-independent vascular movement

Tombusviruses - In the case of *Tomato bushy stunt virus* (TBSV), CP is dispensable for both cell-to-cell and systemic movement (Scholthof *et al.*, 1993). The *Cucumber necrosis virus* (CuNV) dispenses encapsidation for vascular movement, and

most likely does not contain the CP in the viral ribonucleoprotein complex transported via PPU (Sit *et al.*, 1995). Controversially, there are evidences that the CP is necessary for tombusvirus unloading from the phloem, probably in the form of assembled virions (Qu & Morris, 2002). The p19 protein of *Cymbidium ringspot virus* (CymRSV) was found to be essential for virus loading into the phloem, since infection of *N. benthamiana* by a p19-defective mutant was restricted to tissues around the vascular bundle of inoculated leaves (Havelda *et al.*, 2003). Because p19 is the tombusviral suppressor and interferes with the systemic signalling of RNA silencing (Voinnet *et al.*, 1999), it was proposed that p19 prevents the onset of mobile signal-induced systemic RNA silencing ahead of the infection, leading to generalized infection by CymRSV (Havelda *et al.*, 2003).

Umbraviruses - Umbraviruses are exceptional in the sense that they do not encode a CP (reviewed by Syller, 2002). Chimeric TMV derivatives, in which the CP gene was replaced by ORF3 of the umbraviruses *Pea enation mosaic virus-2* (PEMV-2), *Tobacco mottle virus* (TMoV) or *Groundnut rosette virus* (GRV), were capable of systemic spread, demonstrating that ORF3 is a factor involved in the vascular movement (Ryabov *et al.*, 1999; 2001). The ORF3 associates with TMV RNA and forms filamentous ribonucleoprotein particles, but not as uniform as classical virions (Taliansky *et al.*, 2003). It is suggested that similar ribonucleoprotein complexes represent the novel structure that may be used by umbraviruses as an alternative to classical virions and as potential form for umbraviral movement through the phloem (Taliansky *et al.*, 2003). Interestingly, ORF3 does not suppress RNA silencing (Taliansky & Robinson, 2003).

Hordeiviruses - *Barley stripe mosaic virus* (BSMV) is capable of vascular movement in absence of the CP and forms nucleoprotein complexes with the non-structural protein βb (Donald & Jackson, 1996). The BSV γb protein, which is the hordeiviral suppressor of silencing, is also involved in viral systemic movement (Yelina *et al.*, 2002).

For the Tobraviruses *Tobacco rattle virus* and *Pea early-browning virus*, the Pomovirus *Potato mop-top virus* and the Begamoviruses *African cassava mosaic virus* and *Tomato golden mosaic virus* it has been shown that the CP is not required for vascular movement (Etessami *et al.*, 1989; Gardiner *et al.*, 1988; McGeachy & Barker, 2000; Savenkov *et al.*, 2003; Swanson *et al.*, 2002).

Host responses against viral systemic infection

Viruses generally have a limited range of plants in which they can establish an infection successfully, thus all other plants are by definition non host plants. To

accomplish systemic infection of host plants, viruses have to cope with plant defence responses and barriers. According to the type of response, host plants can be broadly categorised as, permissive or semi-permissive. In permissive plants all steps required for systemic infection are supported (i.e. replication, local and systemic spread). If systemic infection is hampered at any step, provided some viral replication still takes place, then the plant is considered a semi-permissive host. The fact that a plant is a semi-permissive host for a virus does not necessarily imply the expression of genes involved in active resistance mechanisms, but can also be the result of absence of host factor(s) indispensable for viral spread.

Responses in permissive host plants

Although CPMV can fully and successfully infect its natural host cowpea as well as its experimental host *Nicotiana benthamiana*, there are a number of antiviral responses and barriers in these hosts with which the virus has to cope during systemic infection. Examples of such defence mechanisms are senescence resistance and post-transcriptional gene silencing (PTGS, also called RNA silencing). Here focus is given on the mechanisms and impact of RNA silencing, a process of sequence-specific degradation of a particular RNA triggered by the presence of its homologous double stranded-RNA (dsRNA) (Yu & Kumar, 2003). In plants, RNA silencing is both an endogenous mechanism for regulation of gene expression in developmental processes and an inherent defence response against viral pathogens, even in permissive host plants (reviewed by Vance & Vaucheret, 2001; Yu & Kumar, 2003). In animals, a similar antiviral function of RNA silencing has not yet been demonstrated.

Upon infection by single-stranded RNA (ssRNA) viruses, the dsRNA that triggers silencing is probably the viral intermediate replicative form, which is recognized as foreign and degraded in a homology-dependent process. The dsRNA that initiates silencing is processed by an RNaseIII-like nuclease (dicer) into short segments of dsRNA 21-23 nt in length, so-called small interfering RNAs (siRNAs) (reviewed by Rovere *et al.*, 2002). Interactions of siRNAs with an RNase complex, named RNA-induced silencing complex (RISC), guides the RISC to degrade RNA species homologous to the siRNAs (reviewed by Rovere *et al.*, 2002). In plants, after the initiation of silencing, a signal is produced that can move between cells as well as systemically through the vascular tissue to direct RNA silencing in the whole plant (reviewed by Mlotshwa *et al.*, 2002a).

To counteract the RNA silencing mechanism plant-infecting viruses encode a suppressor of RNA silencing, which is usually a multifunctional protein also involved in other steps of viral infection (reviewed by Marathe *et al.*, 2000; Li & Ding, 2001). An increasing number of plant viruses appears to encode a protein with suppression

activity (reviewed by Goldbach *et al.*, 2003). Two examples of suppressors are the potyviral HC-Pro protein, being the first recognized viral suppressor of RNA silencing (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998), and the cucumoviral 2b protein (Brigneti *et al.*, 1998).

HC-Pro suppresses one or more maintenance steps in the RNA silencing pathway, beyond the point of initiation of silencing, by inhibiting accumulation of siRNA through an unknown mechanism (Llave *et al.*, 2000) and without interfering with the mobile silencing signal (Mallory *et al*., 2001). Not only does HC-Pro prolong potyviral RNA replication (Kasschau *et al.*, 1997), it also prolongs the accumulation of the heterologous virus PVX (-) strand RNA, thus transactivating PVX replication (Pruss *et al.*, 1997). The effect of HC-Pro in (+) strand RNA accumulation was less dramatic than the effect on (-) strand RNA accumulation (Pruss *et al.*, 1997). Furthermore, two domains spanning the entire central region were described to bind nucleic acids in non-specific fashion, with preference for ssRNA (Maia & Bernardi, 1996; Urcuqui-Inchima *et al.*, 2000), a feature that could be associated with the viral movement and/or suppression of silencing functions of HC-Pro.

The CMV 2b protein was found to produce a distinct pattern of suppression of silencing from that of HC-Pro. CMV 2b could not suppress silencing in leaves where silencing had already been established before virus infection, but it suppressed silencing systemically in upper non-inoculated leaves (Brigneti *et al*., 1998). Thus, in contrast to HC-Pro, the 2b protein suppresses a systemic signalling step in the silencing pathway by blocking transport of the silencing signal (Voinnet *et al.*, 1998; Brigneti *et al*., 1998). 2b encodes a functional nuclear localization signal and nuclear targeting is crucial for its suppressor activity (Lucy *et al.*, 2000; Mayers *et al.*, 2000), suggesting that suppression of silencing may occur in the nucleus (Li & Ding, 2001).

Responses in semi-permissive host plants

Semi-permissive hosts support only part of the viral infection process (e.g. the initial phases). Semi-permissiveness can be based on induced resistance responses or due to the lack of suitable host components required by the virus for infection. Induced resistance often involves the so-called hypersensitive response (HR), by which necrotic lesions are formed as a result of rapid programmed cell death thus containing the viral infection (reviewed by Goldbach *et al.*, 2003). HR is preceded by a specific recognition of the virus (but also fungus or bacteria) by the plant, which is in many cases based on a matching dominant resistance gene (R) and a viral avirulence factor (Avr) (reviewed by Bonas & Lahaye, 2002). Some R genes have been shown to be involved in HR to viral infection, such as the Rx against PVX, HRT against *Turnip crinkle virus*, Sw-5 against *Tomato spotted wilt virus* and N against TMV (reviewed by

Goldbach *et al.*, 2003). In tobacco plants expressing the N resistance gene, many of the inoculated cells undergo HR whereas the systemic non-inoculated leaves become resistant to viral infection by a mechanism denoted systemic acquired resistance (SAR) (Ross, 1961). SAR against TMV involves salicylic acid (SA) signalling (reviewed by Murphy *et al.*, 2001), a plant hormone that also may affect RNA silencing (Ji & Ding, 2001; Xie *et al.*, 2001), viral replication (Chivasa *et al.*, 1997, Naylor *et al.*, 1998) and movement (Murphy & Carr, 2002). The cowpea cv. Early Red and *Phaseolus vulgaris* cv. Pinto show HR against CPMV, but the R gene involved in the recognition is not known.

Plant barriers to systemic infection, specifically impairing virus phloem loading or unloading, have been described for distinct virus-plant systems (Goodrick *et al*., 1991; Wang *et al.*, 1998). In some cases it is known that resistance specifically acting against viral vascular movement is due to recessive genes via an unknown mechanism. Examples are the recessive loci in *N. tabacum* line V20 resistant to TEV (Schaad & Carrington, 1996), the recessive mutation *vsm1* in *Arabidopsis* resistant to *Turnip vein clearing virus* (TVCV) (Lartey *et al.*, 1998), and the *Arabidopsis* recessive gene *va* against PVA (Hämäläinen *et al.*, 2000). In case of *Arabidopsi*s ecotype Columbia, the dominant genes *RTM1* and *RTM2* (Mahajan *et al.*, 1998; Whitham *et al.*, 1999) specifically restrict the vascular movement of TEV without involving HR or SAR. In *N. tabacum*, the cadmium-induced glycine-rich protein (cdiGRP) also blocks the systemic spread of TVCV in a SA-independent manner (Citovsky *et al.*, 1998; Ueki & Citovsky, 2002). *N. tabacum* is an example of a semi-permissive host for CPMV, which does not get systemically infected for unknown reasons.

Outline of this Thesis

Over the past decade, intensive research has supplied detailed information on the mechanisms of CPMV replication and subsequent cell-to-cell movement (see above). However, the mechanisms leading to systemic infection, i.e. viral vascular movement and host responses to the infection are far less understood and were subject of the research described in this thesis.

As at the onset of this thesis research no information was available on the route via which CPMV virus spreads in its cowpea host, in **Chapter 2** infection of different plant tissues, following mechanical inoculation of primary leaves, was recorded as function of time using GFP-expressing recombinant viruses and electron microscopy. In particular the aspects of virus loading into and unloading from the phloem were studied in detail. In **Chapter 3** studies were carried out to determine the form in which CPMV circulates through the vasculature to achieve systemic infection. As viruses face a variety of barriers and host defence responses during infection, in **Chapters 4** and **5** their effect on CPMV infection was analysed. In **Chapter 4**, the impact of RNA silencing on early CPMV infection in the permissive host *N. benthamiana* was examined, while in **Chapter 5** resistance determinants were tested for their involvement in limiting CPMV infection to the inoculated leaf of the semi-permissive host *Nicotiana tabacum*. Since *N. tabacum* supports only virus replication and cell-to-cell movement, this plant species was interesting to mechanistically separate CPMV cell-to-cell movement from vascular movement. Hence several experiments with this plant aimed at a more specific analysis of the systemic spread process alone. Finally, in **Chapter 6** the experimental results described in the previous chapters are discussed and a speculative model for CPMV vascular movement is presented. Moreover, the various virus-host interactions which may occur during virus infection and which contribute to the success or failure of CPMV systemic infection are put into perspective.

CHAPTER 2

Phloem loading and unloading of *Cowpea mosaic virus* in *Vigna unguiculata*

SUMMARY

Within their host plants, viruses spread from the initially infected cell through plasmodesmata to neighbouring cells (cell-to-cell movement), until reaching the phloem for rapid invasion of the younger plant parts (long-distance or vascular movement). *Cowpea mosaic virus* (CPMV) moves from cell-to-cell as mature virions via tubules constructed of the viral movement protein (MP). The mechanism of vascular movement, however, is not well understood. The characteristics of vascular movement of CPMV in *Vigna unguiculata* (cowpea) were examined using GFP-expressing recombinant viruses. It was established that CPMV was loaded into both major and minor veins of the inoculated primary leaf, but was unloaded exclusively from major veins, preferably class III, in cowpea trifoliate leaves. Phloem loading and unloading of CPMV was scrutinised at the cellular level in sections of loading and unloading veins. At both loading and unloading sites it was shown that the virus established infection in all vascular cell types with the exception of companion cells (CC) and sieve elements (SE). Furthermore, tubular structures, indicative of virion movement were never found in plasmodesmata connecting phloem parenchyma cells (PPC) and CC or CC and SE. In cowpea, SE are symplasmically connected only to the CC and these results therefore suggest that CPMV employs a mechanism for phloem loading and unloading that is different from the typical tubule-guided cell-to-cell movement in other cell types.

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INTRODUCTION

To successful infection of host plants, viruses must be able to spread efficiently throughout the plant. Common denominators in this process are (local) cell-to-cell movement through plasmodesmata and utilisation of the phloem transport system to achieve fast systemic spread (long-distance/ vascular movement). After inoculation, plant viruses spread from the infected epidermal cells through the underlying mesophyll cells to the vasculature, to be transported by the phloem to other plant tissues, along with the stream of metabolites (reviewed by Santa-Cruz, 1999). It is well known that plant viruses actively adapt plasmodesmata with the virus-encoded movement protein (MP) to achieve transport of their genome or even virions into neighbouring cells (reviewed by Carrington *et al.*, 1996). Far less is known about the vascular movement of viruses and there appears to be a discrepancy between the strategy used for intercellular movement in leaf mesophyll cells and that used for the entry/exit of the vasculature (reviewed by Séron & Haenni, 1996; Gilbertson & Lucas, 1996; Nelson & van Bel, 1998; Santa-Cruz, 1999; Thompson & Schulz, 1999; Oparka & Santa-Cruz, 2000). In general terms, plant viruses follow the routes of metabolites, from source to sink tissues, and once it has entered into the sieve element, the infectious entity is passively transported to other (sink) plant parts (reviewed by Santa-Cruz, 1999). Hence, vascular movement of viruses apparently is regulated only by mechanisms by which viruses enter (loading) and exit (unloading) from the phloem transport system.

Extensive studies over the past two decades have identified two major strategies for plant virus cell-to-cell movement through plasmodesmata. *Tobacco mosaic virus* (TMV) utilises one strategy, wherein the virus moves in a non-virion form in the absence of coat protein (CP) through plasmodesmata modified by the viral MP (reviewed by Carrington *et al.*, 1996). A second strategy is exemplified by *Cowpea mosaic virus* (CPMV), wherein mature virions are transported through virus-induced tubules that cross the walls of adjacent cells (Wellink & van Kammen, 1989; van Lent *et al.*, 1990, 1991; Kasteel *et al.*, 1993). Several viruses, e.g. potexviruses, need the CP for cell-to-cell movement, but its exact role has not yet been established (Chapman *et al.*, 1992b; Foster *et al.*, 1992, Oparka *et al.*, 1996).

CPMV (reviewed by Goldbach & Wellink, 1996) represents a large group of different plant viruses, including comoviruses (van Lent *et al.*, 1990, 1991), nepoviruses (Wieczorek & Sanfaçon, 1993; Ritzenthaler *et al.*, 1995), caulimoviruses (Perbal *et al.*, 1993) and tospoviruses (Storms *et al*., 1995), which employ the tubule-guided movement mechanism of virions. No information, however, is available on how these viruses are loaded into and unloaded from the plant vascular tissue and which classes of veins are involved in these processes. Other relevant questions are

whether entry into or exit from the sieve element by CPMV also involves a tubule-guided mechanism and in which form the virus is loaded or unloaded (i.e. virion or ribonucleoprotein).

The vascular loading and unloading of several plant viruses have been demonstrated to occur in different patterns in different host-virus systems (e.g. Cheng *et al.*, 2000; Roberts *et al.*, 1997; Sudarshana *et al*., 1998). Since CPMV represents a group of plant viruses with a different cell-to-cell movement strategy, we investigated its vascular loading and unloading characteristics in cowpea (*Vigna unguiculata*) at the macroscopic and microscopic levels. To facilitate this, the green fluorescent protein (GFP) gene was inserted in the CPMV-RNA2 coding region to act as a reporter for virus infection and spread. GFP-expressing recombinant viruses were used to determine the preferred sites (vein classes) for virus loading and unloading. Moreover, veins actively involved in CPMV loading and unloading were analysed for virus pathology at the cellular level.

MATERIALS AND METHODS

Plants and protoplasts

For plant studies, cowpea seeds (*Vigna unguiculata* c.v. California Blackeye) were sown in sterilised soil and grown in a growth chamber at 23 °C with 16 hours light. Plants used for mechanical inoculation had not developed the first trifoliate leaf. For protoplast studies, seeds were germinated in moist vermiculite for 3 days at 27 °C. The germinated seeds were then transferred to hydroponic Hoagland medium and grown in a growth chamber at 23 °C with 16 hours light. Mesophyll protoplasts were isolated from their primary leaves essentially as described by Hibi *et al.* (1975) with modifications described by van Beek *et al.* (1985).

Viral recombinants and inoculations

Two CPMV recombinant viruses, MP19GFP7 and MP19GFP2A, expressing the green fluorescent protein (GFP) through different strategies (Fig. 1) were described earlier (Gopinath *et al.*, 2000). To obtain these recombinants, the GFP gene was inserted into the viral RNA-2 segment within *in vitro* transcription vectors containing the T7 polymerase promoter. Infectious RNA copies of these constructs or a construct containing no GFP sequence (i.e. wild type viral RNA) were made by *in vitro* transcription of the DNA templates. Plasmid DNA templates were purified with midiprep columns (Qiagen). *In vitro* transcription was carried out in 20 µl reactions

using T7 RNA polymerase (Gibco BRL). Each reaction contained 400-500 ng of template DNA, 20 units of RNAse inhibitor (RNAsin, Gibco BRL), 10 units of *Cla*I to linearise the DNA, 1,25 mM of each RNTP (Promega, 25 mM each), 25 units of T7 RNA polymerase and its buffer in adequate final concentration as suggested by the manufacturer. The reactions were incubated at 37 °C for 1-1.5 hours. RNA quantity and quality were checked on agarose gels. *In vitro* transcripts were kept at -20 °C until used as inoculum.

To establish infection, recombinant or wild-type RNA-2 was co-inoculated with *in vitro* transcripts of wild-type RNA-1 (approximately 10 μg of each RNA) onto primary leaves of cowpea plants (usually 8-9 days post sowing) using carborundum powder. Extracts from infected leaf areas containing the recombinant virus were then used for further inoculation experiments.

Alternatively, recombinant virus inoculum was obtained by inoculation of protoplasts with the transcripts. For this, aliquots of 1x 10^6 protoplasts were inoculated with 5 μg each of RNA-1 and RNA-2 *in vitro* transcripts using polyethylene glycol (PEG Mw 6,000) as described by van Bokhoven *et al.* (1993). The protoplasts were then incubated under continuous illumination at 25 °C for 48h and observed for infection using a Zeiss LSM510 laser-scanning microscope. The protoplasts were pelleted and pellets were kept at –20 °C until their use as an inoculum or, for immediate inoculation, 150 μl of phosphate buffered saline (PBS) was added to the pellets and the protoplasts were disrupted by repeated resuspension through a syringe with a high gauge needle.

Electron microscopy

Plant tissues were fixed with 3% (w/v) glutaraldehyde/2% (w/v) paraformaldehyde, 1% (w/v) osmium tetroxide and 1% (w/v) uranyl acetate, dehydrated in ethanol and embedded in London Resin White (LR White, Hard Grade; Electron Microscopy Sciences) essentially as described by van Lent & Verduin (1987). Ultra-thin sections, 70 nm thick, were cut with a diamond knife (Diatome). Prior to gold labelling, sections were treated for 1 h with a saturated solution of sodium metaperiodate (Bendayan & Zollinger, 1983) and washed with distilled water. Immunogold labelling with 10 nm protein A-gold complexes was performed essentially as described by van Lent & Verduin (1986), using rabbit primary antibodies to CPMV particles (van Lent *et al*., 1991), the viral 24 kDa protease (Wellink *et al.*, 1987a) and MP (Wellink *et al.*, 1987b). The gold particles were then enlarged by a silver enhancement using R-GENT SE-LM reagents (Aurion) as suggested by the manufacturer. Finally, sections were stained for 5 min with 2% (w/v) uranyl acetate

and for 1 min with lead citrate (Reynolds, 1963). Specimens were observed with a Philips CM12 transmission electron microscope.

Surgical isolation procedure

The surgical isolation of lamina flaps and midveins of cowpea primary leaves was done essentially as described by Cheng *et al.* (2000) (see also Fig. 3). Plants were inoculated the day after surgical isolation of leaf flaps and midveins (Class I veins) in order to allow the isolated part to recover. The carborundum-dusted lamina flaps and isolated midveins were pinpoint inoculated with infected leaf extract using a flamed Pasteur pipette as described by Wisniewski *et al.* (1990) and Cheng *et al.* (2000). Each inoculated leaf contained 5-10 surgically isolated flaps or one isolated midvein. Only plants with at least one successful pinpoint inoculated spot (based on GFP fluorescence) were included in the experiment. Plants with spots that were fluorescing beyond the cut edge of the flap, or beyond the length of isolated midvein, were discarded. The surgically isolated flaps were detached from the leaf 4 days post inoculation. Experiments were performed twice each for lamina flaps and midveins.

Imaging fluorescence

The spread of CPMV infection was monitored by imaging GFP fluorescence due to recombinant virus accumulation in plant tissue. GFP fluorescence was observed with a Leica stereo fluorescent system consisting of a Wild M3Z stereo microscope equipped with UV illumination and a GFP-plus filter set (excitation 480/40 nm; dichroic beam splitter 505 nm LP; barrier filter 510 nm LP). Images were captured with a CoolSNAP digital camera and processed later. More detailed imaging of infected areas was done using a Zeiss LSM510 laser scanning microscope. GFP fluorescence was observed through excitation with blue laser light at 488 nm and emission through a 505-530 nm bandpass filter. The veinal network, marked by the uptake of red fluorescing Texas Red dextran (Molecular Probes), was simultaneously visualised using green laser light at 545 nm for excitation and emission through a 560 nm longpass filter. To obtain high resolution images of large areas (either EM or LSM images), series of overlapping images were recorded and aligned using the Multiple Image Alignment (MIA) module of the analySIS 3.0 program (Soft Imaging System GmbH, Germany).

RESULTS

GFP-expressing CPMV recombinants are adequate for investigating vascular movement

To facilitate the studies of local and systemic spread of CPMV in cowpea plants, the performance of GFP-expressing recombinant viruses M19GFP7 and M19GFP2A was tested for their suitability. The construction and characteristics of these RNA-2 recombinants and their translational properties are shown in Fig. 1. Both recombinants showed all properties required for tubule-guided movement of virions; i.e. tubular structures containing virus particles were induced at the surface of infected protoplasts and in plasmodesmata of infected plant tissue (data not shown). Furthermore, the recombinant viruses spread locally (cell-to-cell) and systemically in plants, and infection could be traced by GFP fluorescence predominantly in the cytoplasm and nuclei of infected cells. M19GFP7 appeared to be genetically less stable than M19GFP2A, as the virus started losing the GFP gene after about 3 plant passages. With M19GFP2A the recombinant still retained the GFP gene after more than six serial plant passages. In the experiments described here, only second generation recombinant inoculum was used to keep the titre of reverted virus as low as possible (if

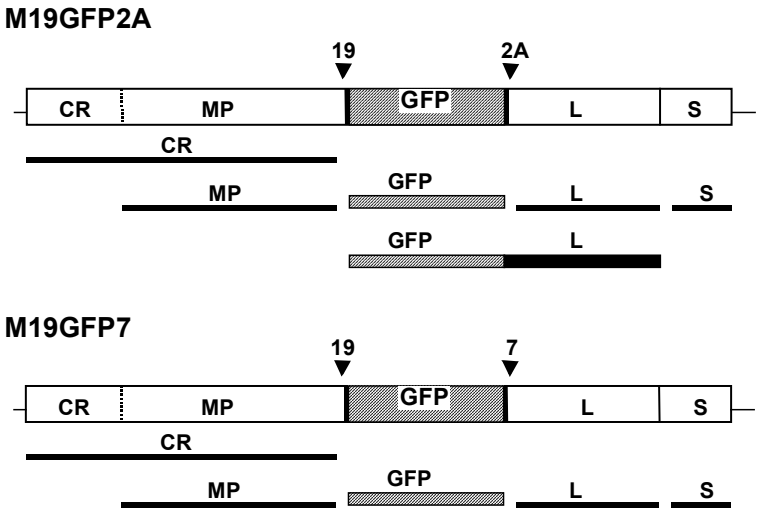

Figure 1. Schematic representation of RNA-2 constructs of the recombinant viruses CPMV M19GFP7 and M19GFP2A and the corresponding expressed proteins. The green fluorescent protein (GFP) gene was inserted between the movement protein (MP) and the large coat protein (L) coding regions in an *in vitro* transcription vector of RNA2 (Gopinath *et al.*, 2000). In M19GFP2A a sequence encoding the foot-and mouth disease virus 2A catalytic peptide was inserted instead of the 7 C-terminal amino acids of the MP. Because of partial cleavage of the 2A site, M19GFP2A expresses free L protein, MP and GFP, but also a GFP-2A-L fusion protein. In M19GFP7, two artificial Q/M proteolytic cleavage sites flanking the GFP gene were created by duplication of a coding region of 19 amino acids from the N-terminus of the L and of 7 amino acids from the MP C-terminus. M19GFP7 expresses free MP, GFP and L. CR, cofactor of replication. S, small coat protein.

at all occurring). In comparable inoculation experiments recording virus spread by appearance of GFP-fluorescence over time, M19GFP7 spread at wild-type speed through the plant, while M19GFP2A appeared to be slightly retarded as infection of upper leaves occurred approximately 12h later when compared to wild-type infection (data not shown). Both recombinants were used in this study: although M19GFP2A had the advantage of genetic stability, M19GFP7 was sometimes preferred because it spread at wild-type speed.

Table 1. CPMV systemic accumulation in cowpea plants over time.

	Days post inoculation													
Plant part*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
a. Roots	-	-	-	+	+	+	+	+	+	+	+	+	+	+
b. Stem below inoculated leaf	-	-	-	+	+	+	+	+	+	+	+	+	+	+
c. Inoculated leaf petiole	-	-	-	-	+	+	+	+	+	+	+	+	+	+
d. Inoculated leaf	-	+	+	+	+	+	+	+	+	+	+	+	+	+
e. Stem above inoculated leaf	-	-	-	-	-	-	-	-	-	+	+	+	+	+
f. 1st trifoliate leaf petiole	-	-	-	-	-	+	+	+	+	+	+	+	+	+
g. 1st trifoliate leaf petiolule	-	-	-	-	-	-	-	-	-	-	+	+	+	+
h. 1st trifoliate leaf	-	-	-	-	+	+	+	+	+	+	+	+	+	+
i. Stem below 2nd trifoliate leaf	N	N	N	N	N	N	N	N	N	-	-	-	-	-
j. 2nd trifoliate leaf petiole	N	N	N	N	-	-	-	-	-	-	-	-	+	+
k. 2nd trifoliate leaf petiolule	N	N	N	N	-	-	-	-	-	-	-	-	-	-
l. 2nd trifoliate leaf	N	N	N	N	-	-	-	-	-	+	+	+	+	+

* Parts of cowpea plant are schematically represented in Fig. 2.
\- Not infected based on GFP fluorescence detection.
\+ Infected based on GFP fluorescence detection.
N Not determined (either not yet developed or too small to be observed).

Kinetics of CPMV systemic spread in cowpea plants

The effect of the developmental stage of cowpea plants on vascular loading and unloading of CPMV was examined. For this, the primary leaves of plants of different developmental stages were inoculated with M19GFP2A or M19GFP7 recombinant viruses and screened for systemic infection by means of GFP fluorescence at 14 dpi (data not shown). Both viruses accumulated in the inoculated leaves regardless of the developmental stage of the plant at the time of inoculation. All tissues of plants were systemically invaded only when inoculated in an early developmental stage, i.e. when the first trifoliate leaf was still folded. On the contrary, when plants of later

developmental stages (i.e. second trifoliate leaf present, no third trifoliate leaf) were inoculated, CPMV failed to accumulate in the first trifoliate leaf, but was unloaded and accumulated in the younger developing upper parts of the plant. Plants already containing the third trifoliate leaf at the time of inoculation supported the replication of CPMV, but no systemic accumulation of the virus was observed. These results demonstrate that the developmental stage of the plant affects CPMV vascular mediated accumulation in cowpea.

To determine the kinetics of CPMV unloading and systemic accumulation, primary leaves of cowpea plants were inoculated with M19GFP7 recombinant virus. The plants used were in a developmental stage permissive to complete systemic infection of cowpea, i.e. the first trifoliate leaf was still folded (9 days post sowing). Plant parts were screened for virus infection at daily intervals for 14 days (3 plants observed per day) (Table 1; Fig. 2). Infection was first observed in the inoculated leaf at 2 days post inoculation (dpi) by the appearance of fluorescent spots which increased in number and size in the following days (Table 1; Fig. 2d, 2d' and 2d"). Systemic spread was first recorded in the stem below the inoculated leaf (Table 1) and in the root (Table 1; Fig. 2a and 2a') at 4 dpi, and infection developed extensively in those tissues the following days (Table 1; Fig. 2b, 2b', 2b'', 2a'' and 2a'''). Remarkably, CPMV was initially transported through the petiole of the primary leaf straight to the stem and roots below the primary leaf without being unloaded in the petiole itself (Table 1; 4 dpi). Only after 5 dpi was infection of this petiole observed (Table 1; Fig. 2c, 2c', 2c'' and 2c'''). Initially, unloading/establishment of infection did not occur in the stem above the primary leaf or in the petiole and petiolule of first trifoliate leaf, but was unloaded in the first trifoliate leaf at 5 dpi (Table 1; Fig. 2h). The petiole of the first trifoliate leaf showed fluorescence only after 6 dpi (Table 1; Fig. 2f and 2f'), whereas infection of tissues in the stem above the primary leaf and in the petiolule of the first trifoliate leaf were first observed, respectively, 10 dpi (Fig. 2e and 2e') and 11 dpi (Fig. 2g and 2g') and onwards (Table 1). Similar to the first trifoliate leaf, the second trifoliate leaf was infected prior (10 dpi onwards; Table 1; Fig. 2l and 2l') to its petiole (13 dpi onwards, Table 1; Fig. 2j and 2j'). Unloading and infection within the stem between the first and second trifoliate leaves and the petiolule of the second trifoliate leaf did not occur in the time span of the experiment (Table 1: i and k). These results demonstrate that CPMV was unloaded and accumulated first in the developing parts of the plant, which are the strongest sink tissues.

To establish how fast CPMV is loaded into phloem and exits from the inoculated leaf, primary leaves of cowpea plants were inoculated with M19GFP7 or M19GFP2A (32 plants for each recombinant) and the inoculated leaves were completely removed at daily intervals up to 7 dpi. At 14 dpi plants were screened for systemic infection.

Systemic infection of the trifoliate leaves was established when the inoculated leaves were removed at 2 dpi or later, but not when the inoculated leaves were removed at 1 dpi (data not shown). These results demonstrate that by 2 dpi CPMV had been loaded into the primary leaf phloem and was transported into the stem.

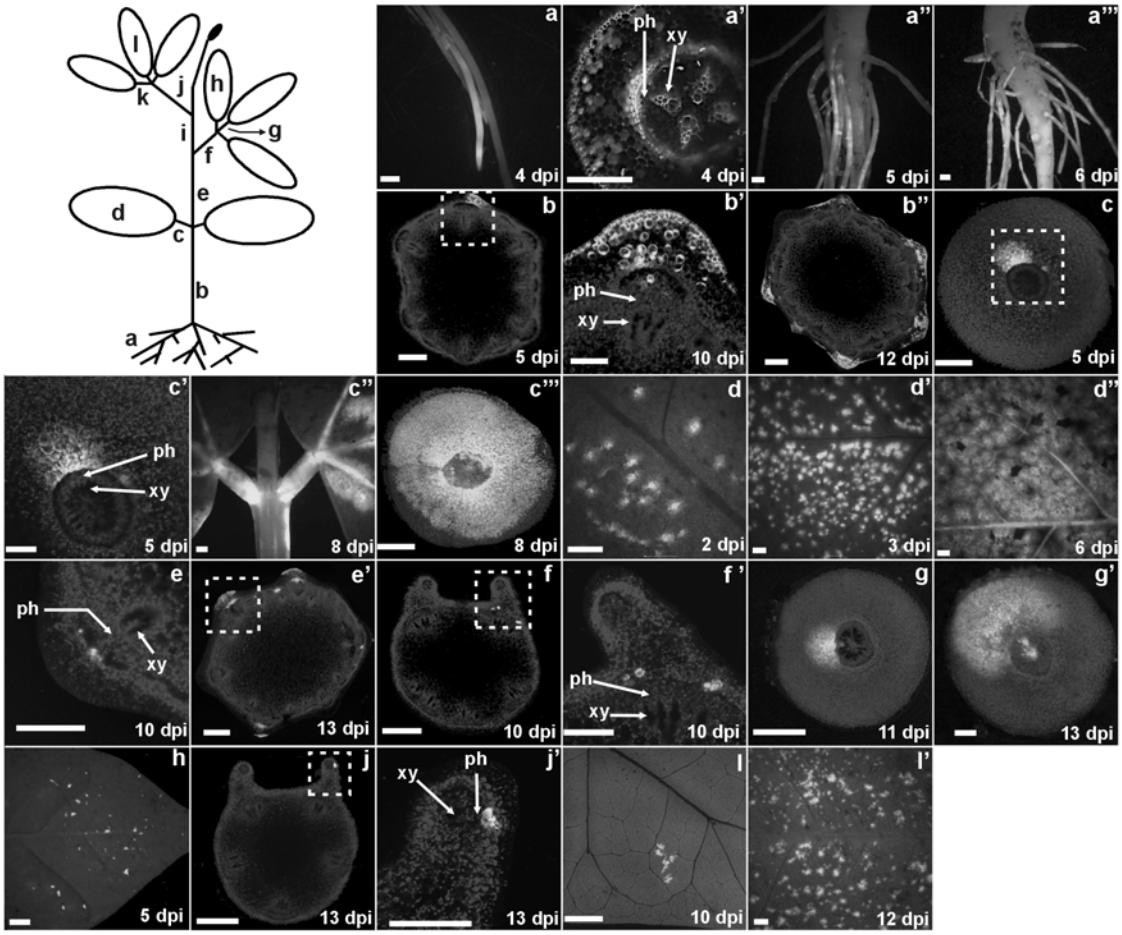

Figure 2. Kinetics of CPMV systemic movement in cowpea plants. Schematic representation of a cowpea plant is at the top left corner of the panel. Fluorescence images depict representative images of CPMV M19GFP7 infection in cowpea over time from 1 to 14 days post inoculation (dpi). Letter identification of fluorescence images corresponds to plant parts shown in the schematic representation of a cowpea plant and Table 1. Ph, phloem. Xy, xylem. Insets in (b), (c), (f) and (j) are depicted in (b'), (c'), (f') and (j'), respectively. Bars represent 200 μm in (b'), (c'), (e), (g') and (i'). Bars represent 500 μm in (a'), (b), (b''), (c), (c''), (e'), (f), (f'), (g) and (i'). In all other images, bars represent 1 mm.

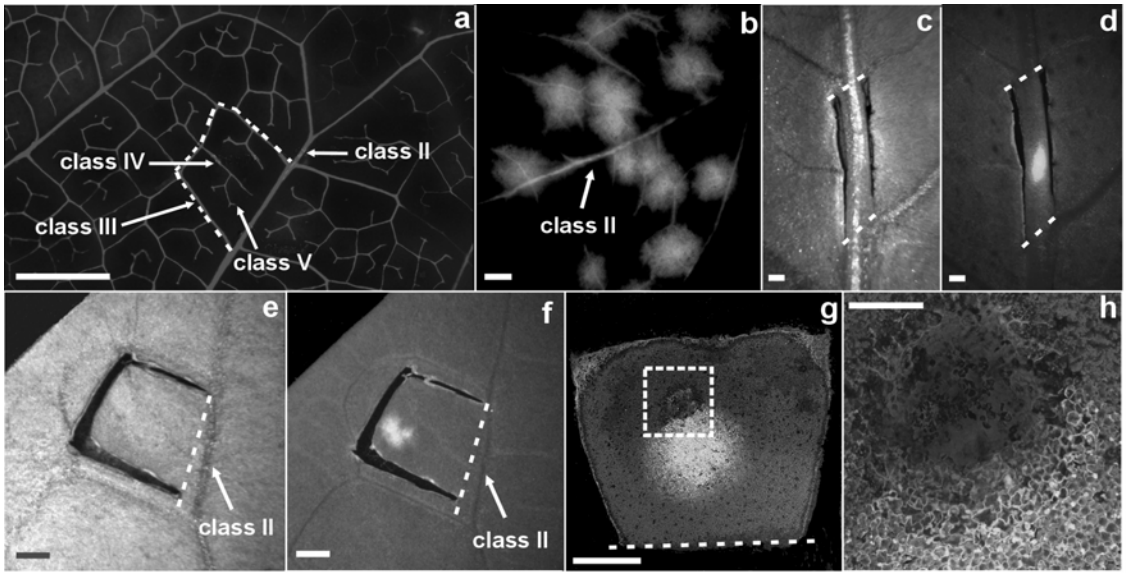

Figure 3. Surgical procedure for determination of loading sites of CPMV in source primary leaves. (a) Veinal structure of a cowpea primary leaf visualised by Texas Red fluorescence. (b) Primary leaf at 4 days after inoculation with recombinant CPMV M19GFP7. (c, d) Brightfield and fluorescent image respectively, of a pinpoint-inoculated isolated midvein at 4 days post-pinpoint inoculation. (e, f) Brightfield and fluorescent image, respectively, of an isolated leaf lamina flap containing only minor veins at 4 days post-pinpoint inoculation. (g) Confocal image of an isolated leaf lamina flap after complete detachment from the plant. (h) Detail of pinpoint-inoculated lesion, corresponding to inset in (g). Dotted lines indicate the detachment sites of both isolated midveins and leaf flaps. Bars represent 200 μm in (h) and 1 mm in all other images.

CPMV is loaded into minor and major veins

For detailed cytological studies of CPMV vascular loading and unloading, it was essential to first establish which classes of veins were involved in these processes. The organisation of the veinal structure of cowpea leaves was visualised by labelling the xylem with Texas Red dextran (Fig. 3a). The veinal network of cowpea plants is organised into a successive branching of veins (Hickey, 1979). From the class I midvein the class II and III veins branch successively (all major veins). Class III veins occur in areoles, inside which minor veins (class IV and V) are present.

Conventional inoculation of GFP-expressing recombinants and subsequent observation of local spread by fluorescence microscopy did not reveal any clue to the class of veins involved in CPMV loading (Fig. 3b). To establish whether there were preferred sites of virus loading, minor veins and major veins were selectively inoculated by means of the surgical isolation procedure described by Cheng *et al.* (2000). Leaf lamina flaps containing only minor veins were surgically isolated from the surrounding major veins with the exception of one side that was left attached to a single class II or class III (major) vein (Fig. 3e). The loading capability of the minor veins was determined by pinpoint-inoculation of the isolated leaf lamina flaps with GFP-recombinant virus. When local spread of CPMV was established (recorded as a

fluorescent spot) but the infected area had not yet reached the connected major vein (Fig. 3f), the flaps were detached completely. The plants were then monitored for systemic infection, indicative of successful virus loading into minor veins, during the following 14 days. Similarly, the loading capability of major veins was studied by pinpoint inoculation of a surgically isolated midvein (Fig. 3c and 3d). Based on the kinetics of CPMV systemic spread reported above, cowpea plants used for the surgical isolation procedure were inoculated in a developmental stage permissive for virus unloading in the first trifoliate leaf and leaf lamina flaps/midveins were removed 4 dpi when the virus had entered the stem. The plants were screened for systemic infection of first trifoliate leaves at 14 dpi.

For each plant, 5 to 10 leaf lamina flaps were inoculated with M19GFP7. After removing the inoculated flaps (4 dpi), each flap was observed for local infection in a confocal microscope (Fig. 3g and 3h). If virus infection on any flap had reached the fresh cut boundary (the site where the flap was attached to the class III vein) the plant was excluded from the experiment. Similar criteria were maintained for pinpoint-inoculation of surgically isolated midveins. The results of these experiments are summarised in Table 2 and demonstrate that CPMV can be loaded into minor veins of cowpea leaves, since approximately one third of the locally infected plants (3 out of 10) became systemically infected. Comparable results were obtained when isolated midveins were pinpoint inoculated as 5 out of 7 locally infected plants became systemically infected after inoculating CPMV onto the isolated midvein (Table 2). These results show that CPMV can be loaded into both minor veins and major veins of cowpea primary leaves to establish systemic infection.

Table 2. Systemic infection of cowpea plants after pinpoint-inoculation of surgically isolated leaf lamina (flap) and midveins.

	Isolated leaf lamina		**Isolated midveins**	
	Local infection 4 days p.i.	Systemic infection 14 days p.i.	Local infection 4 days p.i.	Systemic infection 14 days p.i.
Expt 1	5 / 5 plants* 15 / 27 flaps†	1 / 5 plants	3 / 4 plants 4 / 8 veins	3 / 3 plants
Expt 2	5 / 6 plants 18 / 48 flaps	2 / 5 plants	4 / 4 plants 7 / 7 veins	2 / 4 plants
Total‡	10 / 11 plants 33 / 75 flaps	3 / 10 plants	7 / 8 plants 11 / 15 veins	5 / 7 plants

* Number of plants successfully inoculated and included in the experiment per total number of plants treated.

† Number of isolated leaf lamina flaps that showed infection foci after inoculation per total number of lamina flaps inoculated.

‡ Sum of results from two independent experiments.

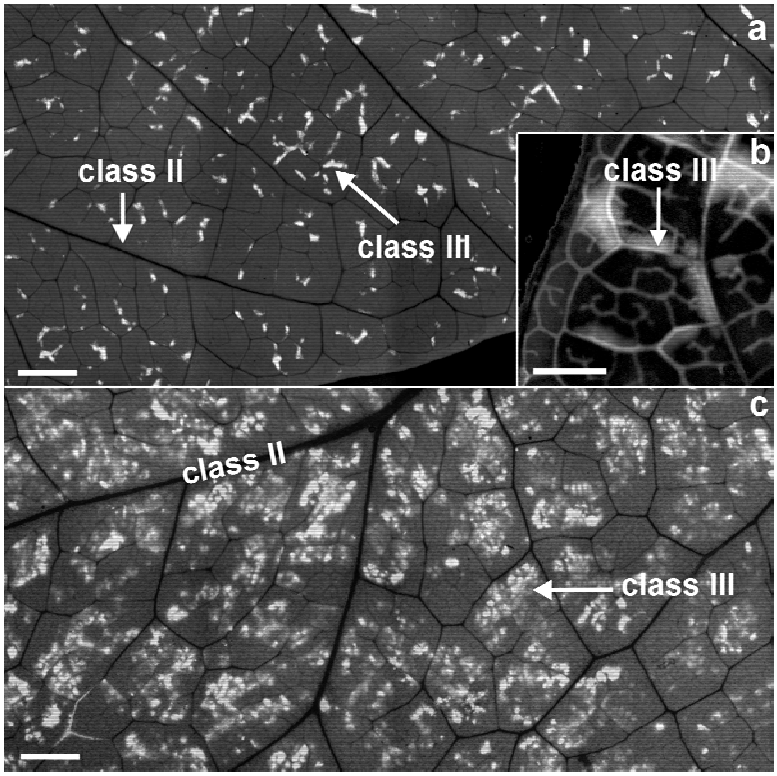

Figure 4. CPMV unloading in the second sink trifoliate leave. Appearance of CPMV M19GFP2A-infected foci on trifoliate cowpea leaves (a, b) at 5 days and (c) 11 days after inoculation of the primary leaves. (b) Inset shows fluorescent foci indicating unloading of virus from a class III vein. The large images were composed by alignment of multiple recordings with a confocal microscope. Bars represent 500 μm in (b) and 1 mm in (a) and (c).

Table 3. Percentage of fluorescing foci associated with different classes of veins in the first trifoliate leaves of M19GFP2A-inoculated cowpea plants at different days post-inoculation (dpi).

Class of vein	5 days p.i.	6 days p.i.	7 days p.i.	10 days p.i.	Mean
I	0	1	1	1	1
II	2	3	2	6	3
III	98	96	97	93	96
Foci *	115	330	566	1040	

* Number of fluorescent foci recorded.

CPMV is unloaded from major veins, preferably class III

To determine the sites of CPMV unloading and accumulation, cowpea plants were mechanically inoculated (9 days post sowing) with M19GFP2A and the first trifoliate leaves were inspected at daily intervals for systemic infection based on GFP fluorescent foci. Systemic infection was recorded by the appearance of fluorescent foci in trifoliate leaves in relation to the veinal structure (Fig. 4a and 4b). At very late stages of CPMV unloading (over 11 dpi) foci were difficult to observe (Fig. 4c).

Fluorescent foci were predominantly associated with class III veins and occasionally with class I and II veins, in particular at later stages of infection, but never with minor veins (Fig. 4b). Table 3 summarises the distribution of a total of 2051 fluorescent foci in relation to the veinal structure, as recorded on trifoliate leaves of three plants at different days after inoculation of the primary leaves. On average, 96% of the veins involved in virus unloading were of the class III type, whereas unloading from class II and class I veins was observed in only 3% and 1% of the cases, respectively. These quantitative analyses demonstrate that all major veins may be used for virus unloading, but that class III veins are overwhelmingly preferred over the other vein types.

CPMV is loaded into and unloaded from sieve elements without apparent replication in companion cells

To identify which vascular cell types were involved in virus loading and unloading, serial sections of veins from M19GFP7- or M19GFP2A-inoculated primary and systemically infected trifoliate leaves were analysed. Target veins were selected by the presence of associated fluorescent foci 3 days post inoculation (dpi) from inoculated leaves and 5 dpi from systemically infected trifoliate leaves. In primary leaves, where the phloem loading occurred, class III and IV veins were excised for microscopical analysis, while in systemically-infected trifoliate leaves, class III veins were analysed. All cells in the vasculature were screened for CPMV replication by the appearance of pathological structures indicative of replication (i.e. electron-dense structures and ER-derived vesiculation) and for virion-containing tubules in plasmodesmata. Furthermore, samples were screened for infection by immuno-labelling of the viral protease, MP and coat proteins.

From each of the three samples of primary leaves and three samples of secondary leaves, five series of five sections were cut, each series at a distance of at least 20 μm from the previous. So in total approximately 75 sections from loading veins (class III and IV) and 75 sections from unloading veins (class III) were analysed, including an estimated number of 15 different sieve element-companion cell complexes for each. As an example, a primary leaf class IV vein screened for CPMV-infection is shown in Fig 5a and 5b. In veins of both primary (source) and trifoliate (sink) leaves, plasmodesmata between bundle sheath cells (BSC) and phloem parenchyma cells (PPC), PPC-PPC (data not shown) and companion cell (CC)-PPC were either linear (Fig. 5e) or branched (Fig. 5f). Connections between sieve element (SE) and CC, in both source and sink leaves, showed the typical structure of a so-called pore-plasmodesma unit (PPU; van Bel & Kempers, 1997), with a single pore on the SE cell wall and branching towards the adjacent CC (Fig. 5c and 5d). Sometimes, SEs showed plasmodesmal connections with more than one CC (Fig. 5c). Apparently, in cowpea

plants the SEs are not symplasmically connected to other cell types but the CC (Fig. 5b and data not shown).

In both loading and unloading veins, CPMV replication was observed by formation of pathologic structures and immunogold/silver staining of viral proteins, in mesophyll cells (MC) and within the phloem only in BSC and PPC (e.g. Fig. 5b). No indications of CPMV-infection could be found in CC or SE (Fig. 5b). In cells of the vasculature, the plasmodesmata were observed for the presence of tubular structures, viral MP and coat proteins, indicative of tubule-guided virion movement. Tubular structures were found at the interfaces MC-BSC, BSC-PPC and PPC-PPC (Fig. 5g and data not shown) of unloading veins, but never between PPC and CC or CC and SE. Also the MP and coat proteins (CPs) antigens were never detected in plasmodesmata of the latter interfaces in either loading or unloading veins (data not shown).

DISCUSSION

In this study we have investigated the characteristics of vascular movement of CPMV in cowpea plants, using GFP expressing viruses to monitor virus infection. Observation of virus infection in cowpea plants overtime, clearly showed that CPMV spreads rapidly from the inoculated primary leaves to the youngest developing plant parts, i.e. roots and developing secondary leaves. This pattern of systemic spread resembles the translocation of photoassimilates from source (primary leaf) to sink (roots and secondary leaves) tissues. The virus did not invade secondary leaves that had fully developed at the time of inoculation. Apparently, as described for *Cauliflower mosaic virus* (CaMV) and photoassimilates in *Arabidopsis thaliana* (Leisner *et al.*, 1992, 1993), CPMV does not invade cowpea leaves via the vasculature after the leaves have passed through the sink-source transition. These results strongly suggest that CPMV is systemically transported through the phloem of the cowpea plant.

By means of a surgical isolation procedure for leaf parts and pinpoint-inoculation of virus it was demonstrated that CPMV can be loaded into the phloem of both major veins and minor veins to establish systemic infection of the upper leaves. Three possible routes for entry of virus into leaf veins have been suggested (Ding *et al*., 1998; Nelson & van Bel, 1998). Viruses could enter the veins at the vein terminus, a gap at a vein branch, or the side of a vein. The successful systemic invasion of cowpea after pinpoint-inoculation of isolated midveins suggests that CPMV is able to approach and enter the phloem stream directly from the surrounding parenchyma tissues. Studies on virus loading into plant vascular tissue are very limited. Recently, Cheng *et al*. (2000) showed that TMV is loaded into minor and major veins and is able to approach and enter the midvein phloem stream directly from the surrounding parenchyma

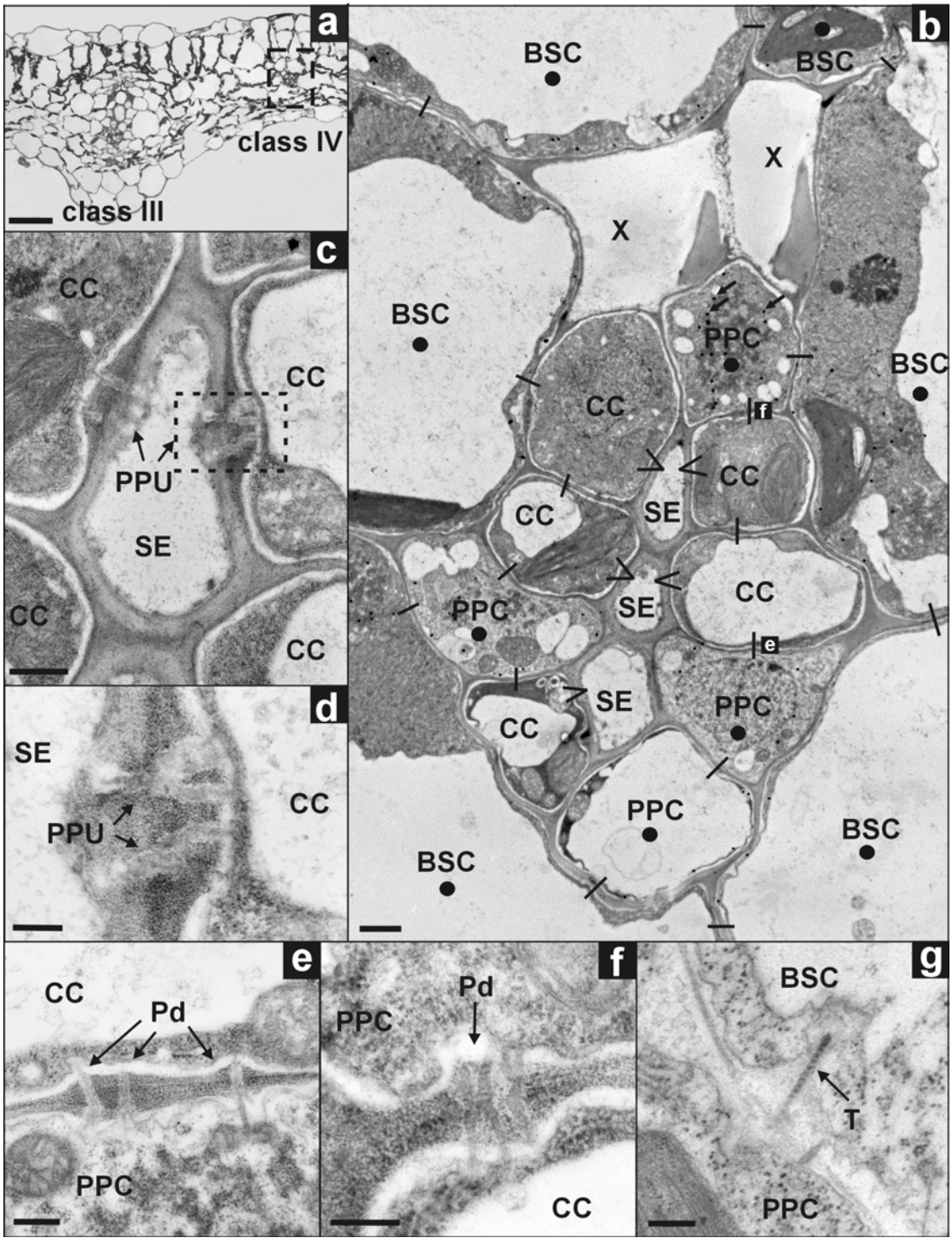

Figure 5. CPMV vascular movement at cellular level. (a) Bright-field image of a cross section of a primary leaf including a class III major and a class IV minor vein. (b) Corresponding EM image of the class IV vein in (a). The lines drawn through cell walls indicate the presence of linear or branched plasmodesmata (single line) or pore plasmodesma-unit (PPU; V-shaped lines). Cells infected with CPMV are marked with a black dot. In this image, CPMV infection was identified by presence of pathological structures and immunogold/silver labelling of viral protease (arrows). (c) Detail of SE-CC symplasmic connections. (d) Magnified view of the inset from (c) showing PPU. (e) and (f) show details of linear and branched plasmodesmata (Pd) in walls between PPC and CC. Their locations are indicated in (b) by corresponding boxed letters. (g) Tubular structure (T) in a plasmodesma connecting BSC-PPC in an unloading vein. X, xylem. BSC, bundle sheath cell. PPC, phloem parenchyma cell. CC, companion cell. SE, sieve element. Bars represent 500 μm in (a), 1 μm in (b), 500 nm in (c), 200 nm in (d), (e), (f) and (g).

tissues. Although plant viruses apparently can be loaded into both major and minor veins, several studies suggest that minor veins are the preferred sites for photosynthate and possibly also for virus loading (reviewed in Nelson & van Bel, 1998).

After phloem transport the virus exits exclusively from major veins and preferentially from the class III veins in the first trifoliate leaves, as over 90% of the fluorescent foci (indicative of CPMV infection) were located adjacent to this vein type. With respect to the preferred sites of phloem unloading and accumulation, CPMV in cowpea shows a similar pattern to that of TMV (Cheng *et al*., 2000), *Tobacco etch potyvirus* (TEV; Oparka & Santa-Cruz, 2000) and *Potato X potexvirus* (PVX; Roberts *et al.*, 1997) in *Nicotiana benthamiana***.** Remarkably, a diverse range of phloem-transported compounds such as radioactive solutes, GFP and systemic RNA signals all exit the phloem exclusively from major veins (reviewed in Oparka & Santa-Cruz, 2000), suggesting that the vein classes used for solute and macromolecule unloading are equally involved in unloading of many plant viruses. Although plant viruses (CPMV, TMV, TEV and PVX) with different mechanisms of cell-to-cell movement show the same vein preference for unloading and accumulation, this does not imply a similarity in the mechanism of unloading at the cellular level.

Careful inspection of serial sections from loading sites in class III/ IV veins and unloading sites in class III veins showed a remarkable absence of CPMV replication (absence of cytopathic structures and viral antigens) in the CC of these vein types. Also, no virions or viral antigens were detected in SEs. However, CPMV replication clearly occurred in the PPC and BSC, besides the epidermal and mesophyll cells. The absence of CPMV replication in CCs in source and sink leaves cannot be explained by symplasmic isolation of the CC-SE complex, as plasmodesmata, though never observed between PPC-SE, were found at PPC-CC, CC-SE as well as at MC-BSC, BSC-PPC, PPC-PPC interfaces. The symplasmic connection between SE via CC with surrounding vascular cells suggests a role of the CC in loading and unloading of photosynthate and also CPMV in cowpea. Absence of virus infection in CCs in inoculated source leaves was observed for *Sunn-hemp mosaic tobamovirus* (SHMV) in *Phaseolus vulgaris* and *Pisum sativum* (Ding *et al*., 1998). For *Potato Y potyvirus* (PVY) and *Peanut stripe potyvirus* (PStV) in *N. benthamiana*, as well as for TMV in *N. benthamiana*, *Capsicum annuum* and *Lycopersicon esculentum,* a preferred infection of vascular parenchyma cells (relative to CC) was found in mature source leaves (Ding *et al*., 1998). It was suggested that some viruses exploit the plasmodesmata between SE and PPC to gain access to the phloem, rather than entering the CCs directly. Considering that no plasmodesmata were ever found between PPC and SE in source leaf cowpea veins, this loading route is less likely for CPMV in this particular host.

For *Cucumber mosaic cucumovirus* (CMV), another spherical virus that is able to form tubules (Canto & Palukaitis, 1999), Blackman *et al.* (1998) reported the presence of virus particles in mature sieve elements in source leaves of *N. clevelandii*. The particles appeared in a membrane-bound viral assembly complex (VAC). Moreover, it was postulated that before CMV is loaded into the SE, virus particles disassemble in the cytoplasm of CC, move through the PPU as a ribonucleoprotein complex and reassemble in the SE (Blackman *et al*., 1998). The fact that no virus was detected in CC could indicate that CPMV might be loaded from CC into SE in a non-virion form.

CPMV cellular localisation in unloading vascular tissue differs from that of PVX (Roberts *et al*., 1997), *Bean dwarf mosaic begomovirus* (BDMV) (Wang, H.-L. *et al.*, 1996) and SHMV (Ding *et al.*, 1998), which were detected in the CC of sink leaves of systemically infected plants. PVX was detected in CC and occasionally in immature SE of *N. benthamiana* sink leaf veins, but vascular parenchyma cells were more heavily infected than CCs. BDMV was detected in CCs of systemically infected *P. vulgaris* leaves, but not in SEs. In systemically infected leaves of *P. sativum*, SHMV viral aggregates were detected in both vascular parenchyma cells and CCs of minor veins. In contrast to what is known for PVX in tobacco, BDMV in bean and SHMV in pea plants, CPMV was apparently unloaded from cowpea leaf veins without replicating in the CC.

The observation of virion-containing tubules in plasmodesmata between the MC-BSC, BSC-PPC and PPC-PPC interfaces in unloading veins shows that CPMV is capable of moving through some phloem cells by means of the well-described mechanism of tubule-guided cell-to-cell movement. Interestingly, tubular structures or virus particles were never observed in the PPU connecting SE-CC of cowpea infected leaves, in source or in sink tissues. The presence of virus particles in the cavity of PPUs was reported for *Carrot red leaf luteovirus* in *Anthriscus cerefolium* (Murant & Roberts, 1979), *Potato leafroll luteovirus* in potato (Shepardson *et al.*, 1980) and *Beet western yellows polerovirus* in sugarbeet (Esau & Hoefert, 1972), in *Thlaspi arvense* (D'Arcy & Zoeten, 1979) and in *N. clevelandii* (Mutterer *et al.*, 1999). Several studies have indicated that PPUs may allow the passage of large molecules (Kempers *et al.,* 1993; Kempers & van Bel, 1997; van Bel, 1996; Turgeon, 2000). For the monocot *Triticum aestivum*, plasmodesmal channels involved in SE/CC unloading can be exceptionally large with a physical diameter of as much as 42 nm (Fisher & Cash-Clark, 2000). Since the PPUs in several plants have a large size exclusion limit, it might be possible that CPMV is loaded into and/ or unloaded from cowpea veins without gating or modifying the PPU. Whether the phloem loading and unloading of CPMV involves transportation of a virion or ribonucleoprotein complex, and whether the MP or other viral proteins play a role in this process remains to be determined.

ACKNOWLEDGEMENTS

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CHAPTER 3

Evidence that *Cowpea mosaic virus* virions are systemically translocated through the vasculature of plants

SUMMARY

Cowpea mosaic virus (CPMV) cell-to-cell movement involves virion transport through tubular structures built-up from the virally encoded movement protein and which penetrate the plasmodesmata. The mechanism of CPMV systemic spread through the vasculature (vascular movement) is still obscure, but seems not to involve tubule-guided loading of virions into the phloem sieve element. The viral form by which CPMV circulates through the vasculature of infected plants has now been investigated. Immunoblot analysis of vascular sap collected from infected cowpea plants revealed the presence of the viral coat proteins only. Furthermore, virions were found in the vasculature of immune cowpea scions grafted on CPMV-inoculated susceptible rootstocks, indicating that CPMV circulates as virions to infect plants systemically. Possible mechanisms of CPMV vascular movement are discussed.

INTRODUCTION

Within the phloem stream of higher plants there is a diverse range of compounds such as nutrients, low-molecular-weight solutes and macromolecules, in dynamic circulation from source to sink tissues (Santa-Cruz, 1999). Most plant viruses have developed strategies to use the same route, i.e. from source to sink tissues, to spread systemically in their host plants by circulating through the phloem along with the assimilate stream (Samuel, 1934; Leisner *et al.*, 1992; Leisner & Turgeon, 1993; Leisner *et al.*, 1993; Lucas & Gilbertson, 1994; Roberts *et al.*, 1997). Several viruses have been shown to require the coat protein (CP) for movement through the phloem, moving systemically either as virions or as viral nucleoprotein complexes (reviewed by Carrington *et al*., 1996). For some other viruses, the CP is dispensable for systemic spread and these viruses move in a non-virion form, i.e. nucleoprotein complex, through the vasculature (reviewed by Santa-Cruz, 1999). *Tobacco mosaic virus* (TMV), for instance, requires the CP for efficient systemic spread throughout the plant (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Ding *et al*., 1996), although for local spread the CP is dispensable (reviewed by Carrington *et al.*, 1996). It has been suggested that the CPs must assemble into virions to enable vascular movement of TMV virus particles along the phloem (Esau & Cronshaw, 1967; Saito *et al.*, 1990). Virions of *Cucumber mosaic virus* were also observed in the phloem sieve element of infected plants (Blackman *et al*., 1998).

For *Cowpea mosaic virus* (CPMV), a beetle-transmissible virus (reviewed by Gergerich & Scott, 1996) consisting of spherical virions, which encapsidate the bipartite single stranded RNA genome (reviewed by Pouwels *et al.*, 2003), only limited information about its mechanism of systemic spread is available. Local cell-to-cell spread has been more extensively studied and involves the translocation of mature virions through tubular structures which are composed of viral movement protein (MP) and are assembled inside modified plasmodesmata (van Lent *et al*., 1990, 1991; Wellink & van Kammen, 1989). However, Silva *et al.* (2002) showed that loading of CPMV into sieve elements, the onset of systemic spread, probably does not involve tubule-guided movement of virions.

It has been suggested that beetle-transmissible viruses, like CPMV, are directly delivered into the xylem by the insect vector, thus using the xylem as the prevailing route for systemic spread within the plant (reviewed by Gergerich, 2001). However, upon mechanical inoculation of primary leaves of cowpea, CPMV is loaded into minor and major veins and subsequently spreads through the phloem in a similar pattern as assimilates do, i.e. unloading in the secondary leaves occurs predominantly from Class III veins (Silva *et al*., 2002).

Here experiments aimed at further elucidation of the route of CPMV systemic movement and the form in which the virus circulates through the vasculature are described. CPMV constructs encoding the green fluorescent protein (GFP) were used to facilitate the investigations in the CPMV-cowpea system.

MATERIALS AND METHODS

Plants and virus inocula

Vigna unguiculata (cowpea) cv. California Blackeye (CBE) and cowpea line TVu-470 (TVU) seeds were sown in sterilised soil and grown in a growth chamber at 23 °C with 16 hours light. Transgenic *N. tabacum* cv. Burley 21 line KM8 (Nida *et al.*, 1992) expressing the 60 kDa precursor of CPMV coat proteins, and line MON4 (Nida *et al.*, 1992) transformed with the empty binary vector pMON530, were a gift from Dr. Said Ghabrial, Department of Plant Pathology, University Kentucky, Lexington-US. *Nicotiana benthamiana* plants were used for agroinfiltration experiments. Wild type *Cowpea mosaic virus* (CPMV) inoculum was maintained in CBE by mechanical inoculation onto primary leaves of plants at 9 days post sowing (dps). The CPMV wild type RNA1 construct and the GFP-expressing RNA2 constructs used are available in *in vitro* transcription vectors under the control of the T7 RNA polymerase promoter. The RNA1 construct was described by Eggen *et al.*, 1989. The RNA2 mutant MGFPΔCP, in which the coat proteins was replaced by the GFP gene, was described by Verver *et al.*, 1998. The RNA2 constructs M19GFP7 and M19GFP2A was described by Gopinath *et al.*, 2000. *In vitro* transcriptions from the plasmid templates were performed as described by Silva *et al.* (2002). Co-inoculation of *in vitro* transcripts of RNA1 and MGFPΔCP result in CPMV-ΔCP mutant virus. Co-inoculation of *in vitro* transcripts of RNA1 and M19GFP7 or M19GFP2A result in viruses (here after referred to as M19GFP7 or M19GFP2A, respectively) that move systemically in permissive hosts for CPMV. Extracts from CPMV-GFP infected cowpea leaves were then used to further amplify the inoculum in cowpea. Wild type CPMV virions were purified from infected cowpea leaves as described by van Kammen (1967) except for the sucrose gradient step that was omitted.

Mechanical and gross-wound inoculation

Mechanical inoculations were performed onto carborundum-dusted primary leaves at the adaxial surface. *In vitro* transcripts of RNA1 and RNA2 MGFPΔCP were co-inoculated mechanically onto *N. benthamiana* leaves as described by Silva *et al.* (2002), just prior to agroinfiltration of the leaves with the pMONCCP60 construct. *In*

vitro transcripts of RNA1 and M19GFP7 were co-inoculated mechanically onto cowpea leaves as described by Silva *et al.* (2002). For gross-wound inoculation, ice cold freshly prepared inoculum solution containing 1 mg/ml purified M19GFP7 virus particles and 200 μg/ml bovine pancreatic RNase A (Sigma) in PBS pH 7.2 was used. The gross-wound inoculations were performed (based on Gergerich *et al.*, 1983; 1991) by punching primary leaves of CBE plants (9 dps) at the adaxial surface, onto 6-10 non-overlapping sites per leaf, with a hollow glass cylinder of 1 cm diameter that had just been dipped into the inoculum solution. Inoculated plants were then incubated in growth chamber under conditions described above.

Fluorescence microscopy

CPMV infection was monitored by imaging GFP fluorescence in plant tissue with a Leica stereo fluorescent system consisting of a Wild M3Z stereomicroscope equipped with UV illumination and a GFP-plus filter set (excitation 480/40 nm; dichroic beam splitter 505 nm LP; barrier filter 510 nm LP). More detailed imaging of infected areas was done with a Zeiss LSM510 laser scanning microscope. GFP fluorescence was detected by excitation with blue laser light at 488 nm and emission through a 505-530 nm bandpass filter.

Collection of vascular sap

CBE cowpea plants used for collection of vascular sap were inoculated with M19GFP7 at 9 dps, before the first trifoliate leaves had developed. Vascular sap (i.e. phloem plus xylem sap) samples were collected at 7 days post inoculation (dpi), when the first trifoliate leaf was unloading the virus (unloading observed based on GFP fluorescence). Plants were topped with a razor blade at the stem above the primary leaves, immediately under the first trifoliate leaf (see schematic representation of cowpea plant in Fig. 1i). Vascular sap was collected from the acropetal side of the sectioned stem. The first droplet of vascular sap was wiped off with filter paper to avoid contamination of cell contents of the non-vascular surrounding tissue. The following droplet (about 5 μl per plant) was immediately collected with automatic micropipette and mixed with 5 μl of ice-cold vascular extraction buffer (0.2 M Tris HCl pH 8.0; 10 mM EDTA; 40 mM DTT; based on Golecki *et al.*, 1999). Samples were maintained on ice until immediate separation on SDS-PAGE. After collection of vascular sap samples, the lower and upper cut edges of the stem were screened for GFP fluorescence indicative of viral replication and infection out the vascular tissue. Only samples from stems that were not presenting any infection in the tissues surrounding the vascular bundles were used for the analysis. Samples from several

plants were pooled and analysed by SDS-PAGE. Vascular sap samples from healthy plants were collected as described for infected plants.

SDS-PAGE and Immunoblot

Infected and healthy CBE primary leaves were ground in vascular extraction buffer (0.1 g fresh tissue/ml), spun briefly to remove debris and the supernatant was collected and for SDS-PAGE analysis. Protein samples were separated by SDS-PAGE in 12% polyacrylamide gels at pH 8.8 (Laemmli, 1970) in a Bio-Rad minigel system and either stained with Coomassie Brilliant Blue R-250 or blotted onto Immubilon-P membranes (Millipore), using a semi-dry TransBlot apparatus (Bio-Rad) and Tris-glycine buffer (Towbin *et al.*, 1979). The membranes were blocked overnight in 3% low-fat milk powder in Tris-buffered saline (TBS: 50 mM Tris-HCl, 200 mM NaCl, pH 7.4) containing 0.1% Tween-20 (TBS-Tween). Membranes were washed three times 5 min. in TBS-Tween and incubated for 1 hour with primary antibodies diluted 1:2000 in TBS-Tween containing 0.1% low-fat milk powder. Membranes were washed two times 10 min and two times 5 min. in TBS-Tween and incubated for 1 hour with secondary donkey-anti-rabbit antibodies conjugated with horseradish peroxidase (Amersham) diluted 1:5000 in TBS-Tween containing 0.1% low-fat milk powder. Subsequently, membranes were washed two times 15 min. and three times 5 min. in TBS-Tween and incubated with chemiluminescent substrate for peroxidase (ECL, Amersham) for 1 min. before exposure to film (X-omat AR, Kodak).

Antibodies

Rabbit polyclonal antibodies (Ra) used to probe CPMV proteins were against: CPMV (this serum detects large (L) and small (S) coat proteins) (van Lent *et al*., 1991), 24K protease (Wellink *et al.*, 1987a), MP (Wellink *et al.*, 1987b) and 32K cofactor for the protease (Franssen *et al.*, 1984). Chicken polyclonal antibodies (Cha) against CPMV were obtained from eggs of chicken immunized with purified CPMV particles. Immunoglobulins were purified from egg yolks as described by (Polson *et al.*, 1980). Rabbit polyclonal antiserum against barley rubisco (ribulose-1,5-bisphosphate carboxylase-oxygenase) was a gift of Dr. Klimentina Demirevska-Kepova (Bulgarian Academy of Sciences – Sofia, Bulgaria; Demirevska-Kepova *et al.*, 1993).

Grafts

CPMV-susceptible cowpea CBE plants at 16-20 dps were used as rootstock in all cases. Plants used as rootstocks had fully developed primary leaves and secondary leaves that were not yet fully expanded. CBE or CPMV-immune cowpea TVU plants were used as scions at 10 or 12-13 dps, respectively, when plants had no expanded secondary leaves yet. Both primary leaves of the rootstock plants were inoculated with either wild type CPMV or M19GFP2A just prior to grafting. Rootstock plants were topped at the stem about 5 cm above the primary leaves and cut in a straight incision into the stem end. The scions were obtained by cutting plants at the stem about 7 cm below the primary leaves and carving a V-shaped tip at the stem end. The graft union was sealed with tape. After grafting, the scions were immediately covered with transparent plastic bags containing a wet piece of cotton to prevent tissue desiccation. Grafted plants were incubated in growth chamber under conditions as described above. The plastic bags were partially opened at 7 days post grafting and completely removed at 11 days post grafting. The presence of virus in the rootstock or scion leaf midvein extracts was investigated 14-20 dpi by immunosorbent electron microscopy and by bio-assay (i.e. mechanical back inoculation onto CBE plants).

Intergrafts

In intergrafts, CBE rootstock and scion were connected by a TVU stem graft. CBE rootstock and scion were prepared as described before. For the TVU intergrafts plants were cut about 3 cm below and above the primary leaves. The grafting procedure was performed as described above. The presence of virus in the rootstocks, intergrafts and scions was investigated 14-20 dpi by back inoculation of leaf midvein or stem extracts onto CBE plants.

Immunosorbent electron microscopy (ISEM)

ISEM was performed on 400 mesh carbon-coated nickel grids. The grids were incubated for 30 min. at room temperature (RT) on 20 µl droplets of Ra-CPMV, diluted 1:100 in PBS. Grids were washed 3 times 1 min. on PBS droplets and subsequently incubated overnight at 4 °C on droplets of sample to (immuno)trap CPMV virions. Grids were then washed 3 times 1 min. with PBS and negatively stained with 2% uranyl acetate or alternatively incubated on Cha-CPMV (10 µg/ml in PBS-BSA 1%) for 1 hour at RT for further immunogold labelling of CPMV virions. After washing 6 times 1 min. with PBS, grids were incubated for 1 hour at RT on rabbit-anti-chicken antibodies conjugated with 6 nm gold particles (Aurion), diluted 1:40 in PBS-BSA 1%. Grids were washed 3 times 5 min. with PBS and negatively

stained with 2% uranyl acetate. Specimen were air-dried and observed with a Philips CM12 transmission electron microscope.

Agrobacterium T-DNA transient-expression assay (ATTA)

The constructs used for Agrobacterium T-DNA transient-expression assay, containing genes encoded by the CPMV RNA2, were available either in the binary vector pMON530 or pBIN19 and express the various proteins under the control of the 35S promoter: 60 kDa precursor of coat proteins (pMONCCP60, Nida *et al.*, 1992; gift from Dr. Lomonossoff, John Innes Centre, Norwich, UK.), 58 kDa cofactor for RNA2 replication (pBINM58/48, Sijen *et al.*, 1995) and 48 kDa MP (pBINM48, Sijen *et al.*, 1995). All binary constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404, except for pMONCCP60 which was introduced into the strain GV3103 (also denoted strain PMP90). Each agrobacterium culture was grown overnight at 28 °C in 2 ml LB-3 medium (1% peptone; 0.5% yeast extract; 0.4% NaCl; 0.1% KCl; 0.3% $MgSO_4$) containing 20 μg/ml of rifampicin and 50 μg/ml of kanamycin (or 300 μg/ml of streptomycin, in the case of pMONCCP60) as selection markers for agrobateria and binary constructs, respectively. The cells were pelleted by centrifugation for 5 min at 2250 *g* and each culture was resupended in 10 ml of induction medium [1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% trisodium citrate, 1 mM $MgSO_4$, 0.2% (w/v) glucose, 0.5% (v/v) glycerol, 10 mM MES (pH 5.6) and 50 μM acetosyringone]. After overnight incubation at 28 °C in induction medium, cells were pelleted again and washed in 10 ml Murashige-Skoog (MS) medium [4.4 g/l MS salts (Duchefa); 3% sucrose; pH 5.8] containing 10 mM MES (pH 5.6). Cells were resuspended to a final OD_{600nm} of 0.5 in MS medium-MES with 150 μM acetosyringone and these suspensions were used in ATTA (also referred to as agroinfiltration). The complete area of young fully expanded *N. benthamiana* leaves was pressure-infiltrated with the bacterial suspensions from the abaxial leaf surface using a 0.5 ml needle-less syringe. Agroinfiltrated plants were covered with transparent plastic for 2 days and kept in a growth chamber at 23 °C with 16 hours light.

RESULTS

‘Gross-wound inoculation’ versus mechanical inoculation with carborundum

CPMV is a beetle-transmissible virus thought to use the xylem as the significant route for vascular movement upon vector inoculation (Gergerich, 2001). However, upon mechanical inoculation, CPMV uses the phloem, not the xylem, as the route for

systemic spread (Silva *et al.*, 2002). To get an insight whether the xylem and/or phloem is the natural route for CPMV vascular movement, gross-wound inoculation was used. Gross-wound inoculation (Gergerich *et al*., 1983) has been suggested to mimic virus transmission by beetles because (i) the presence of RNase in the inoculum provides conditions similar to that found in beetle regurgitant and (ii) gross wounding itself imitates the beetle feeding damage caused in the plant vascular system (Gergerich *et al.*, 1991). Gross-wound inoculation is thought to enable virus entry into the xylem where the virus is then transported away from the wound edge (Gergerich, 2001). To assess whether gross-wound inoculation could result in delivery of CPMV into the phloem of leaves, cowpea plants were gross-wound inoculated with the GFP-expressing recombinant CPMV M19GFP7. At 6 dpi, M19GFP7 had spread locally throughout mesophyll cells adjacent to the gross-wound inoculation edge (Fig. 1a, 1b and 1c). In several of the inspected gross-wound edges threads of cells that seem to belong to the vasculature (either phloem or xylem) showed virus derived fluorescence (Fig. 1d and 1e), indicating that M19GFP7 was apparently delivered into the vascular tissue at the gross-wound site. At 10 dpi, M19GFP7 had spread from the site of inoculation (Fig. 1f) to the midvein, possibly through the vasculature instead of by cell-to-cell movement (note pattern of spread in Fig. 1g). Furthermore, at 10 dpi, 4 out of 7 gross-wound inoculated plants were systemically infected and the infection pattern resembled that of phloem-mediated transport, i.e. from source to sink tissues (Fig. 1h, 1i, 1j and 1k). These observations demonstrate that, independently from the possibility of CPMV delivery into xylem, gross-wound inoculation mimicking of beetle transmission resulted in delivery of CPMV into the phloem and subsequent systemic spread to sink tissues. Moreover, these results indicate that the phloem, rather than the xylem, is the prevailing route for CPMV vascular movement. In this respect, gross-wound inoculation does not differ from classical mechanical inoculation by means of an abrasive. Therefore in all following experiments mechanical inoculation onto carborundum-dusted leaves was used.

CPMV coat proteins are the only viral factors detected in vascular sap

To investigate which viral factors are systemically transported along the vascular stream in CPMV-infected (M19GFP7) cowpea plants, samples of vascular sap from several infected plants were collected and pooled for immunoblot analysis.

Since sap was collected from severed stems at the first internode (Fig. 1i), it contained both phloem and xylem contents. After sampling, the cut stem surfaces were inspected for GFP-fluorescence (indicative of viral replication) to assure that no CPMV-infected cells were present in the stem tissues (as virus from such cells would contaminate the sap sample). To check whether the vascular sap collected had

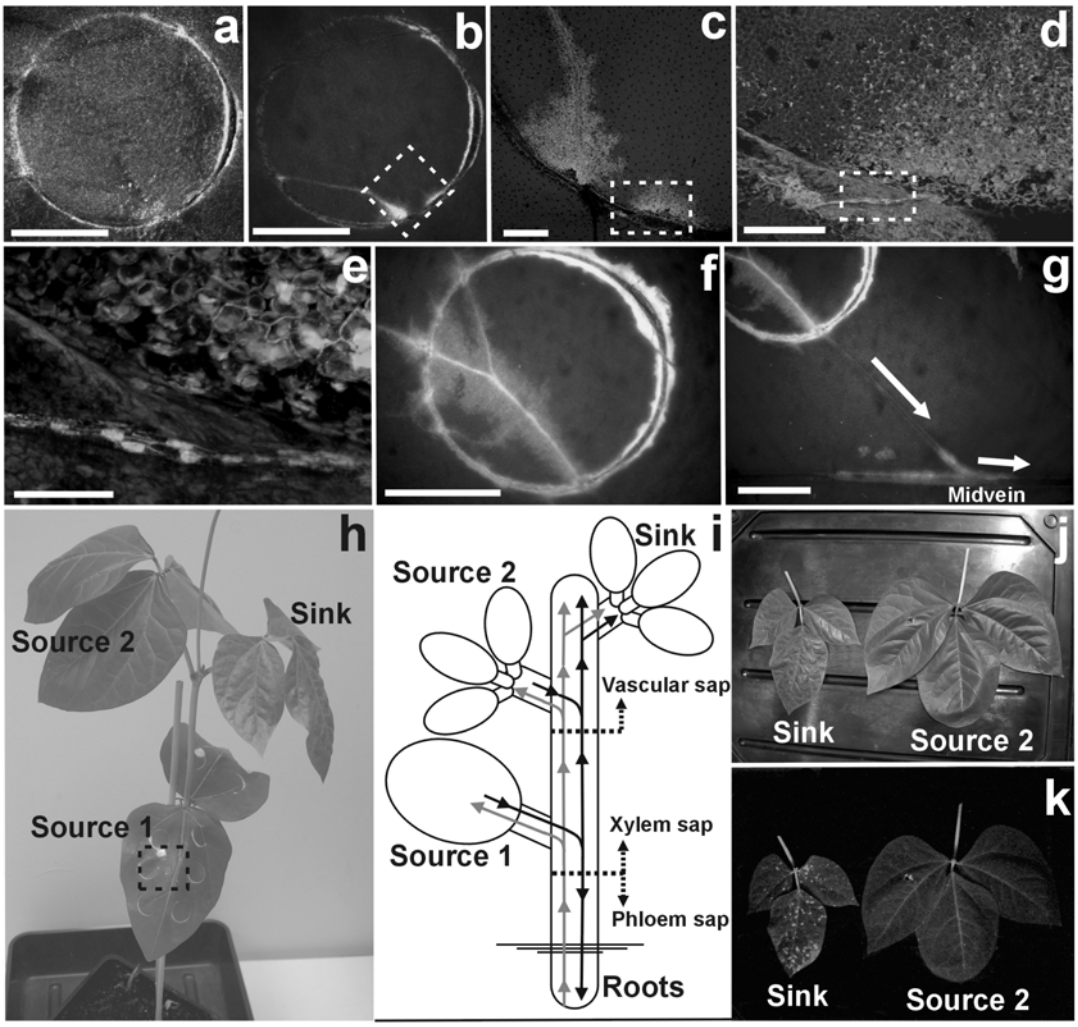


Figure 1. Gross-wound inoculation of cowpea plant. (a) Brightfield and (b-e) fluorescence images of a site of gross-wound inoculation with GFP-expressing CPMV (M19GFP7) at 6dpi; (b) to (e) are successive enlargements of the insets. (f, g) Fluorescence image of the same site in (b), at 10dpi. Note viral infection (fluorescence) originated from gross-wound inoculation site spreading towards leaf midvein. (h) Bright field image of the plant gross-wound inoculated on 'Source 1' leaf. Inset is the site shown in detail in the previous images. (i) Source-sink relations in the plant in (h). Note that xylem stream (grey arrows) is ascendant from the roots to the aerial parts of the plant, whereas the phloem stream (black solid arrows) flows from source to sink tissues. Vascular-, xylem- and phloem-sap (dotted lines) indicate sites where the corresponding sampling was performed. See text for details of the sampling. (j) Bright field and (k) fluorescence images of 'Sink' and 'Source 2' leaves from plant in (h). Note that only the 'Sink' leaf became systemically infected (GFP-fluorescence) with virus coming from the 'Source 1' gross-wound inoculated leaf. Bars represent 0.5 cm in (a), (b), (f) & (g); 1 mm in (c); 200 μm in (d); 100 μm in (e).

contaminating contents of non-vascular cells, the samples were analysed in Western blots for the presence of rubisco, a protein that is totally absent in the vascular tissue. Both the large and small subunits of rubisco were easily detected in extracts from leaves (Fig. 2a) and stem (data not shown), but not in the collected vascular sap fraction (Fig. 2a) indicating that contamination with non-vascular cell content was neglectable.

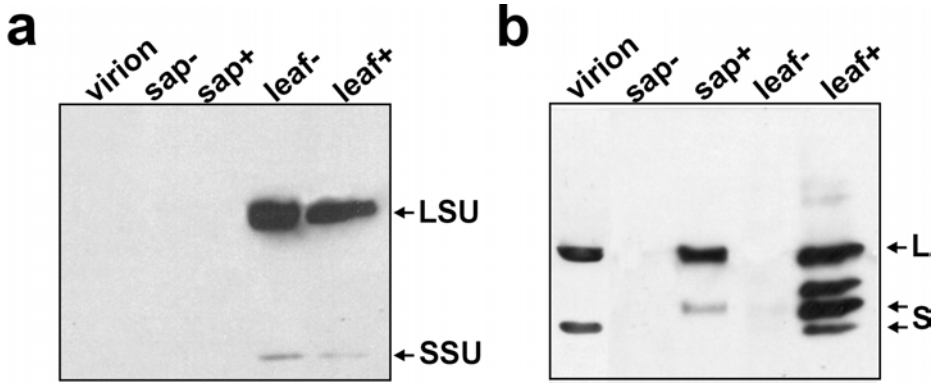


Figure 2. Detection of CPMV proteins in vascular sap of infected cowpea. (a) Immunoblot probed with anti-rubisco. (b) Immunoblot probed with anti-CPMV. virion: CPMV purified virus particles. sap-: vascular sap from healthy cowpea plant. sap+: vascular sap from CPMV-infected cowpea plant. leaf-: extract from healthy cowpea leaf. leaf+: extract from CPMV-infected cowpea leaf. L, CPMV large coat protein. S, slow and fast running forms of CPMV small coat protein. LSU, rubisco large subunit. SSU, rubisco small subunit.

Similar blots were probed with different antibodies against CPMV structural and non-structural proteins. Blots probed with antiserum against CPMV showed the presence of both L and S CPs in vascular sap and leaf extracts from infected cowpea plants (Fig. 2b). The MP, the viral protease (24K) and the cofactor for the protease (32K) were not detected in vascular sap samples (data not shown). As the detection of the CPs in vascular sap could reflect the presence of CPMV virions, similar vascular sap samples were tested in ISEM for the presence of virus particles. However, despite scrutinous inspection of many EM specimen, no particles were found (data not shown).

To discriminate whether the CPs detected in the vascular sap originated from phloem and/or xylem, three different approaches were used to separately collect phloem and xylem sap. Xylem sap was collected from the acropetal side of the stem severed below infected primary leaves (Fig 1i) using the $CaCl_2$-method described by Schobert & Komor (1990). Phloem sap was collected from the basipetal side of the stem severed below an infected primary leaves using the EDTA-method described by King & Zeevaart (1974) and Lejeune *et al.* (1988). Aphid honeydew, which represents the phloem sap content, was collected as described by Wilkinson *et al.* (1997) from insects that fed on infected primary leaves. In none of these samples, however, structural or non-structural viral proteins could be detected (data not shown). Probably, the amount of viral proteins was too low to be detected by immunoblotting.

Virions accumulate in the vasculature of grafted CPMV-immune cowpea plants

To determine if the CPs detected in vascular sap of CPMV-infected plants are in a virion form or not, CPMV-immune (i.e. does not support virus replication *in planta*) cowpea cv. TVu-470 (TVU) (Sterk & de Jager, 1987) scions were grafted onto

CPMV-susceptible cowpea cv. California Blackeye (CBE) rootstock, and the latter was inoculated with wild type CPMV. Approximately two weeks later, extracts from the midveins of secondary leaves of the TVU scions were tested for infectivity by inoculation onto the primary leaves of CBE plants (bio-assay) and for the presence of CPMV virions by ISEM using anti-CPMV antibodies. The bio-assays revealed that in nearly all (22 out of 24) TVU scions, CPMV was present in an infectious form, while the scions showed no symptoms of virus infection (Table 1). In similar samples from the TVU scions, virions were consistently found by ISEM (Table 1; Fig. 3a). As CPMV does not replicate in TVU (Sterk & de Jager, 1987) the virions must have been transported from the infected rootstock to the scion through the vascular system. Controls consisted of grafts with CBE scions on CBE rootstocks and always scored positive in bio-assays and in ISEM (Table 1; Fig. 3b). Similar grafts were infected with CPMV-M19GFP2A instead of the wild type virus and the surfaces of hand-made sections through the midveins were inspected for GFP fluorescence (indicative of virus replication). Also here, infected cells were never found in TVU scions.

CBE scions, interconnected to wild type CPMV-inoculated CBE rootstock by a TVU intergraft, became systemically infected (Table 1). Since CPMV does not replicate in TVU, these results show that, once the virus is loaded in the vasculature, virus replication is not required for vascular transport of CPMV.

Table 1. Grafts and intergrafts using CPMV-susceptible and -immune cowpea plants

Rootstock[a]	**Intergraft**	**Scion**	**Total nr of grafts**	**Symptoms**[c]	**Bio-assay**[d]	**ISEM**[d]
CBE[b]	-	CBE	16	16	10(10)	10(10)
CBE	-	TVU	31	0	22(24)	16(17)
CBE	TVU[b]	CBE	6	5	-	-
Plant						
TVU[e]	-	-	14	0	0(8)	0(6)

(a) Rootstock leaves were inoculated with wild type *Cowpea mosaic virus* (CPMV).
(b) CBE, *Vigna unguiculata* (cowpea) cv. California Blackeye, susceptible to CPMV. TVU, Cowpea cv. TVu-470, immune to CPMV.
(c) Typical CPMV symptoms on sink leaves of scion (or non-grafted TVU control) plants.
(d) Between brackets the total number of scions (or non-grafted TVU) sink leaves tested.
(e) Control plants; not grafted.

Taken together, the results obtained demonstrate that CPMV circulates through the vasculature of infected plants as virions, which do not replicate along the vascular stream.

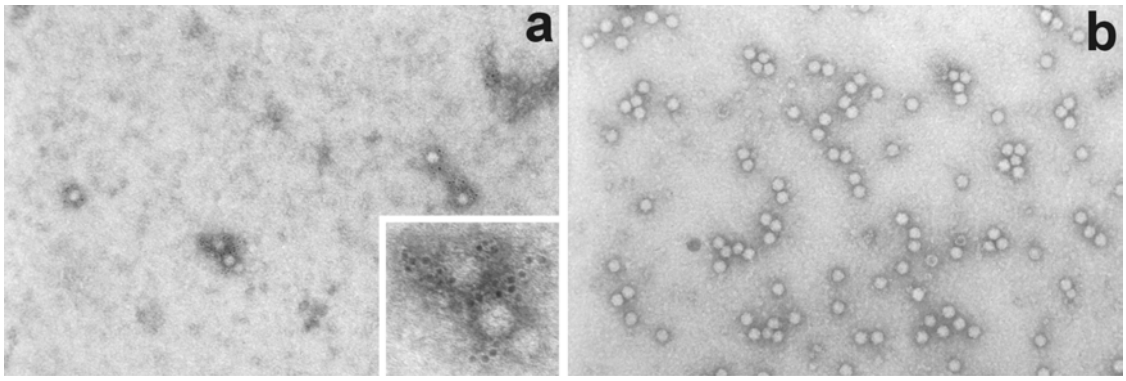
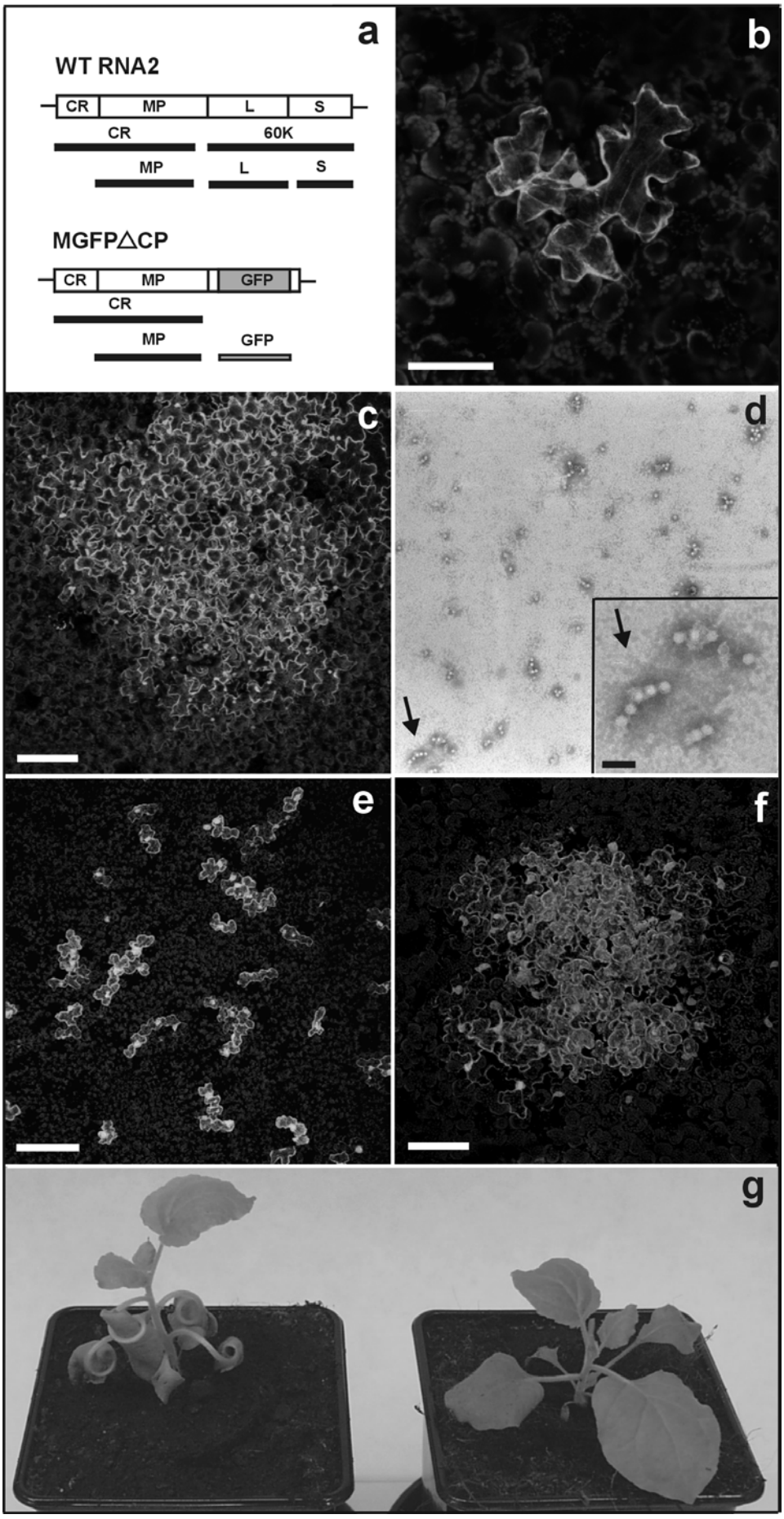


Figure 3. ISEM analysis of leaves from grafted cowpea cultivars. (a) ISEM of leaf midvein extracts from cowpea leaf of immune cv. TVu-470 (TVU) scion grafted on susceptible CPMV-infected cv. California Blackeye (CBE) rootstock depicts virions. Inset displays immunogoldlabelling of virions with anti-CPMV serum. (b) ISEM of leaf midvein extracts from cowpea leaf of the CBE scion grafted on CPMV-infected CBE rootstock depicts virions.

Reverse genetics approach to investigate involvement of CPs/MP in CPMV vascular movement

In an ultimate attempt to assess whether CPs or MP are involved in CPMV vascular movement, CPMV GFP-expressing mutants depleted of either the CPs (CPMV-ΔCP) (Fig. 4a) or MP (CPMV-ΔMP) (obtained by co-infection of wild type.

Figure 4. CPMV-ΔCP infection in absence or presence of the 60 kDa precursor of CPMV coat proteins (CPs). (a) Schematic representation of CPMV wild type RNA2 (WT RNA2) and mutant RNA2 (MGFPΔCP) lacking the coding region for the 60 KDa precursor of coat proteins (CPs). Co-inoculation of CPMV wild type RNA1 with MGFPΔCP generates the mutant virus CPMV-ΔCP. CR, 58 kDa cofactor for RNA2 replication. 60K, 60 kDa precursor of CPs. MP, movement protein. L, large CP. S, small CP. GFP, green fluorescent protein. (b) CPMV-ΔCP is restricted to a single cell in *N. tabacum* line MON4, which is transformed with empty binary vector pMON530 (3 dpi). The leaf was inoculated with viral *in vitro* transcripts. (c) CPMV-ΔCP local spread complemented in *N. tabacum* line KM8 transformed with binary vector pMONCCP60, which expresses the 60 kDa precursor of CPMV CPs (3dpi). The leaf was inoculated with viral *in vitro* transcripts. (d) ISEM of infection foci from plant in (c) depicts CPMV-ΔCP virions. The arrow indicates a detail magnified in the inset. (e) Back inoculation of leaf extracts of infection foci from plant in (c) onto cowpea plants depicting infectious CPMV-ΔCP virus with limited local spread (3 dpi). (f) CPMV-ΔCP local spread complemented in *N. benthamiana* leaf agroinfiltrated with the construct pMONCCP60. The leaf was inoculated with leaf extracts containing infection foci from plant in (c), which contained CPMV-ΔCP virions. Bars represent 100 nm in (d), 100 μm in (b) and 200 μm in (c), (e) and (f). Distorted phenotype of plant agroinfiltrated with CPMV MP construct. (g) *N. benthamiana* plant agroinfiltrated with pBINM48 construct encoding the MP (left) or with pBINM58/48 construct encoding the CR (right); 3 days post agroinfiltration.


a
WT RNA2
CR
MP
L
S
CR
60K
MP
L
S
MGFP△CP
CR
MP
GFP
CR
MP
GFP
b
c
d
e
f
g

RNA1 and RNA2 MGFPΔMP *in vitro* transcripts; Verver *et al*., 1998) was used to observe virus phloem unloading in absence of these factors. The fact that CPMV requires both CPs and MP for cell-to-cell movement implies that the mutant viruses must be complemented *in trans* within the inoculated leaf in order to attain the vascular tissue for phloem loading. Once loaded into the phloem, the phloem unloading of the mutant viruses was to be observed in sink leaves, where no complementation *in trans* was provided. Transgenic lines of CPMV-permissive hosts expressing the CPs or the MP were not available. Alternatively these proteins where delivered for *in trans* complementation by agroinfiltration of the inoculated leaf of permissive host *N. benthamiana*. Agroinfiltration of cowpea leaves was inefficient and therefore not suitable (data not shown). *In vitro* transcripts of CPMV-ΔCP were initially used as inoculum. In the absence of *in trans* complementation CPMV-ΔCP is limited to a single cell-infection in both *N. benthamiana* (not shown) and in *N. tabacum* (Fig. 4b). The efficiency of inoculation with *in vitro* transcripts is low (Chapter 4 of this dissertation). Therefore, virions of CPMV-ΔCP were obtained by inoculating the transgenic line KM8 of the semi-permissive host *N. tabacum* expressing the precursor of the CPs. CPMV-ΔCP cell-to-cell movement was complemented in KM8 plants (Fig. 4c). Extracts of CPMV-ΔCP infection foci from KM8 plants contained encapsidated CPMV-ΔCP virions (Fig. 4d) that were infectious when inoculated onto cowpea plants (Fig. 4e). In *N. benthamiana*, agroinfiltration with a construct encoding the precursor of the CPs (pMONCCP60) and inoculation with CPMV-ΔCP virions resulted in successful *in trans* complementation of cell-to-cell movement (Fig. 4f). Nevertheless, local spread was not extensive enough to reach the vascular tissue and CPMV-ΔCP was thus not loaded into the phloem, consequently phloem unloading of the mutant could not be recorded. Agroinfiltration of *N. benthamiana* with a construct encoding the MP (pBINM48K), to complement the phloem loading of CPMV-ΔMP, resulted in a morphologically disturbed phenotype of curling leaves, petioles and stem (Fig. 4g). Despite the fact that the cofactor for RNA2 replication (CR) and the MP coding regions overlap (Fig. 4a), agroinfiltration with a CR-expressing binary vector (pBINM58) did not result in curling phenotype (Fig. 4g). The complementation of CPMV-ΔMP cell-to-cell movement was not performed because of the impediment of the drastic curling phenotype.

DISCUSSION

CPMV systemic infection is the result of cell-to-cell movement of mature virions from infected cells to neighbouring uninfected cells through plasmodesmata, until the vasculature is reached from where a so far unknown infectious entity is rapidly

transported to other plant parts. To reach the vasculature virions move by a tubule-guided mechanism from the mesophyll via endodermal and phloem parenchymal tissues to the companion cell (CC)-sieve element (SE) complex (van Lent *et al*., 1991; Silva *et al*., 2002). The nature of the infectious entity subsequently loaded into the SE is not known, but tubules were never found at the interfaces between phloem parenchyma and CCs or CCs and SEs (Silva *et al.*, 2002). Here it was demonstrated that the CPs were the only viral factors detected within the vascular sap of CPMV-infected plants. Moreover, results from TVU/CBE grafts showed that mature virions were loaded into the vascular stream of the infected CBE rootstock, and reach the vasculature of developing leaves at the immune TVU scion. The presence of CPMV CPs in vascular sap of infected cowpea and virions in midvein extracts of grafted TVU scions may indicate that, like in cell-to-cell movement, virions are transported along the phloem stream to establish systemic infection. However, it cannot be completely excluded that virions detected in TVU scions originated from xylem vessels. CPMV may have reached the xylem stream by infecting undifferentiated xylem cells in the roots of the CBE infected rootstock. Upon maturation of the xylem vessels virions may then be released in the water stream and reach aerial parts of the plant (French & Elder, 1999), *in casu* the TVU scion.

Systemic spread through xylem has been suggested for beetle transmissible viruses (reviewed by Gergerich, 2001). Experimental evidence for this came from gross-wound inoculation, a technique that mimics beetle transmission (Gergerich *et al.*, 1983; 1991). However, here it is shown that upon inoculation, either mechanically using an abrasive or by gross wounding inoculation, CPMV systemic spread occurs via the phloem and directed to sink-leaves only, thus following the route of photo-assimilates. In view of these results, it is probable that in nature beetle transmission will also result in CPMV systemic infection of plants via the phloem.

For several other viruses it has been shown that the CP is essential for movement through the phloem and/or that virions or viral ribonucleoproteins complexes circulate along the vascular stream. For TMV, which does not require the CP for cell-to-cell movement (reviewed by Carrington *et al*., 1996), the CP is necessary for systemic spread through the plant (Takamatsu *et al.*, 1987; Dawson *et al.*, 1988; Ding *et al*., 1996) and it was suggested that virions move along the phloem stream (Esau & Cronshaw, 1967; Saito *et al.*, 1990). Controversially, Dorokhov *et al*. (1983, 1984) suggested that TMV ribonucleoprotein complexes built up of viral RNA and CP may, besides virions, also be a viral form systemically transported within tobacco. The CP of *Potato virus X potexvirus* (PVX) moves along the phloem, but there is no information available on the viral form that moves systemically in the plant (Santa-Cruz *et al*., 1998). *Red clover necrotic mosaic dianthovirus* (RCNMV) requires virion

formation for systemic infection of plants (Vaewhongs & Lommel, 1995). *Cucumber mosaic virus* (CMV) somewhat resembles CPMV in that the virus also employs a tubule-guided mechanism for intercellular transport of virions (Suzuki *et al.*, 1991; Canto *et al.*, 1997). A discrete mutation in the N-terminal region of the CMV CP still permits cell-to-cell movement but vascular movement is abolished (Suzuki *et al*., 1991), indicating the CP plays a role in vascular movement. Evidence that CMV spreads systemically in a virion form is the detection of CP and virions in mature sieve elements (Blackman *et al*., 1998). It was postulated that virus particles disassemble in the CC and move as ribonucleoprotein complex through the plasmodesmata to the SE, where they reassemble into virions (Blackman *et al*., 1998).

As CPMV cell-to-cell movement requires both CPs and the MP, employing reverse genetics to investigate CPMV factors involved in vascular movement presents major technical challenges as mutation of CPMV genes either compromises replication or affects already cell-to-cell movement of the virus. In an attempt to circumvent these limitations, an *in trans* complementation approach was used. To investigate the role of CPs in vascular movement, local spread of a CPMV-ΔCP mutant (lacking the CPs) was complemented by providing the 60 kDa precursor of the CPs *in trans* via agroinoculation. The *in trans* expression of the 60 kDa precursor successfully complemented CPMV-ΔCP local spread. However, the CPMV-ΔCP local spread (complemented *in trans*) was apparently not extensive enough for the virus to reach the vascular tissue of the inoculated leaf, thus phloem unloading of the mutant could not be studied. It was reported that when S and L CPMV CPs were expressed from separate promoters, the 60 kDa precursor was dispensable for virion formation in both cowpea protoplasts (Wellink *et al.*, 1996) and insect cells (Shanks & Lomonossoff, 2000). Our observations demonstrate that in the absence of S and L encoded proteins instead, *in trans* expression of the 60 kDa precursor results in generation of infectious virus particles *in planta* (Fig. 4c-4e). Reverse genetics could still be a valid approach to investigate CPMV vascular movement if the *in trans* complementation is provided by a transgenic line that constitutively expresses the required protein.

It seems feasible that virions of CPMV and other viruses are loaded into/unloaded from the SE through the specialized plasmodesmata called plasmodesma-pore units (PPU) that are present in the CC-SE cell wall. For several plant species it has been reported PPU allow the passage of exceptionally large molecules (Kempers *et al.,* 1993; Kempers & van Bel, 1997; Turgeon, 2000; Fisher & Cash-Clark, 2000). Although CPMV is able to move from cell-to-cell as virions by means of tubule-guided cell-to-cell movement, tubules or virus particles were never observed in the PPU between SE and CC in infected cowpea leaves (Silva *et al.*, 2002). The potential high size exclusion limit (SEL) of PPU may allow passage of mature virions into the

SE without the modification of these plasmodesmata, in contrast to intercellular movement through mesophyll plasmodesmata. To test this possibility, it would be important to determine the SEL of PPU of permissive host plants for CPMV.

Viral factors other than the CPs may be crucial for vascular movement of several viruses. The CMV 3a MP was demonstrated to move through PPUs of *N. tabacum* (Itaya *et al.*, 2002). Also indirect evidence is available for the involvement of the TMV replicase gene or protein in vascular movement (Holt *et al*., 1990; Ding, X.S. *et al*., 1995). Though able to move from cell-to-cell and reach the SE, a *Tobacco etch potyvirus* (TEV) mutant in the helper-component proteinase (HC-Pro) was impaired in further vascular movement (Cronin *et al*., 1995). Thus, HC-Pro is essential for either transport of TEV within the SE or for virus unloading from the phloem. The MP is indispensable for cell-to-cell movement of plant viruses (reviewed by Carrington *et al*., 1996), but its role in vascular movement is mostly not determined, and in the case of TMV it is even controversial (Gera *et al.*. 1995; Arce-Johnson *et al.*, 1997). For CPMV the MP is able to bind viral RNA *in vitro* (Carvalho, *et al.*, 2004), but as the MP was never detected in vascular sap of infected plants it is unlikely that a ribonucleoprotein complex involving the MP is the phloem-transported infectious form responsible for systemic infection.

Immunoblots only revealed the presence of the L and S CPs in vascular sap, but not that of viral proteins involved in replication (24K protease and its precursors, and the 32K cofactor). This and the observation that susceptible CBE scions, interconnected to CBE rootstocks by immune TVU intergrafts, became infected demonstrate that, like TMV (Susi, 1999), CPMV does not depend on replication in the vasculature during vascular transport.

In summary, the results presented here suggest that CPMV employs a mechanism for phloem loading and unloading that is different from the typical tubule-guided cell-to-cell movement. The presence of virions in the vasculature suggests that, like for cell-to-cell movement, also vascular movement occurs in this form. However, transport of a non-virion form, i.e. viral RNA complexed with CP or MP can not be excluded yet. Further investigations are necessary to unequivocally determine the infectious form transported by the phloem and to establish the role of other viral and host factors in phloem loading and unloading of CPMV in cowpea.

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CHAPTER 4

Role of RNA silencing in establishment of *Cowpea mosaic virus* infection

SUMMARY

The potential role of RNA silencing during establishment of infection by Cowpea mosaic virus (CPMV) was studied. Using GFP-expressing viral constructs and *Nicotiana benthamiana* as host, the number of infection foci was recorded in the absence or presence of different viral suppressors of RNA silencing, i.e. potyviral HC-Pro, tospoviral NSs and cucumoviral 2b. Using CPMV in vitro transcripts as inoculum HC-Pro, either transgenically expressed or provided by agroinfiltration, increased the number of CPMV primary infection foci significantly. A similar effect was observed with tospoviral NSs but not with cucumoviral 2b protein. These results indicate that RNA silencing already influences the establishment of infection at a very early stage. Surprisingly, the stimulating effect of viral suppressors of gene silencing was not observed when virions were used as inoculum. To assess whether RNA silencing also plays a modulating role during subsequent local spread from a primary infection site, GFP-expressing CPMV constructs impaired in local spread were tested in the presence or absence of HC-Pro or NSs. Neither of these proteins affected the progress of infection, indicating that RNA silencing seems not to play a major role in this stage.

INTRODUCTION

Infection of host plants by viruses is the outcome of a competition between viral replication and spread and host anti-viral defence. Plants have developed mechanisms like hypersensitive response (HR), which limits the viral infection to a local lesion (for recent review: Goldbach *et al.*, 2003) or RNA silencing, which results in sequence-specific degradation of viral RNA (for recent reviews see Rovere *et al.*, 2002; Baulcombe 2002; Yu & Kumar, 2003), to arrest virus infection. RNA silencing is induced by double stranded RNA (dsRNA) (Waterhouse *et al.*, 1998) and results in sequence specific breakdown of homologous RNA molecules. For ssRNA viruses, dsRNA present as the replicative intermediate formed during replication is the putative target of a Dicer-like dsRNA specific nuclease (Bernstein *et al.*, 2001), which cleaves it into small interfering RNAs (siRNAs) (Hamilton & Baulcombe, 1999). These siRNAs subsequently guide a homology-dependent degradation of viral RNA by cytoplasmic nucleases denoted RNA-induced silencing complex (RISC) (Hammond *et al.*, 2001). To counteract the plant RNA silencing mechanism, viruses in turn encode suppressors of silencing, which usually are multifunctional proteins involved in various steps of viral infection (reviewed by Marathe *et al.*, 2000; Li & Ding, 2001).

Here we have studied the impact of RNA silencing during the onset of virus infection using *Cowpea mosaic virus* (CPMV) and *N. benthamiana* as model system. CPMV has its genome divided into two positive sense ssRNA segments denoted RNA1 and RNA2. Its expression strategy is based on the production of polyproteins, which are proteolytically processed into several intermediate and final cleavage products. The proteins involved in replication are encoded by the RNA1, whereas the RNA2 encodes for proteins involved in cell-to-cell movement, i.e. the large (L) and small (S) coat proteins (CPs) and the movement protein (MP) (reviewed by Pouwels *et al.*, 2002a). Cell-to-cell movement of CPMV occurs through tubular structures built up from MP copies that penetrate plasmodesmata to mediate cell-to-cell movement of whole virions (Wellink & van Kammen, 1989; van Lent *et al.*, 1990; Kasteel *et al.*, 1993). The vascular movement of CPMV is less understood and seems to involve a mechanism other than the tubule-guided cell-to-cell movement (Silva *et al.*, 2002). There are several steps in the CPMV infection process where RNA silencing can potentially affect the success of virus infection. Like with other plant viruses, also for CPMV an activity of suppression of silencing was shown, though relatively weak and observed only in systemically infected tissue (Voinnet *et al.*, 1999). The impact of suppression of RNA silencing on the success of virus infection has been shown for several plant viruses (reviewed by Marathe *et al.*, 2000; Li & Ding, 2001), but it is not known whether suppressors also play an early role in initiation of CPMV infection. Especially for RNA viruses, it can be envisaged that RNA silencing, as an early host

response, may already be operable in the initially infected cell. To test this, the CPMV infection frequency and extent in mechanically inoculated *Nicotiana benthamiana* leaves was monitored in the presence or absence of different heterologous suppressors of RNA silencing. For easy recording of number and size of the infection sites, GFP-producing constructs of CPMV were used.

MATERIALS AND METHODS

Plants and viral constructs

Seeds of *Nicotiana benthamiana*, non-transgenic or HC-Pro-expressing transgenic line h44 (Mlotshwa *et al.*, 2002b) were sown in sterilised soil and plants were grown in a growth chamber at 23 °C with 16 hours light. Approximately one month-old tobacco plants with five fully expanded leaves were used for agroinfiltration and inoculation experiments.

Wild type and recombinant CPMV were maintained in *Vigna unguiculata* var. California Blackeye (cowpea). Cowpea plants were grown under the same conditions as described for tobacco plants and were inoculated 8-9 days post sowing.

All CPMV constructs used were cloned in *in vitro* transcription vectors under the control of the T7 RNA polymerase promoter. CPMV RNA1 construct was described by Eggen *et al.* (1989). The CPMV RNA2 constructs M19GFP7 and M8GFP2A were described by Gopinath *et al.* (2000). The CPMV mutant MGFPΔCP (hereafter referred to as M29GFP-ΔCP), in which the CPs were replaced by the GFP gene, was described by Verver *et al.* (1998). To generate the M19GFP7-F8 construct, the GFP coding region was included in the original CPMV construct named FMDV-VIII (Porta *et al.*, 2003). For that purpose, the *Sst*I fragment of the FMDV-VIII plasmid (containing a FMDV epitope insertion into CPMV small coat protein) was used to replace the corresponding region in the construct M19GFP7, resulting in the construct M19GFP7-F8.

Agrobacterium T-DNA transient-expression assay

The constructs used for Agrobacterium T-DNA transient-expression assay (ATTA) were available in the binary vector pBIN19 and express the various proteins either under the control of the 35S promoter, in case of GFP (Bucher *et al.*, 2003), *Cowpea aphid borne mosaic virus* (CABMV) HC-Pro (Mlotshwa *et al.*, 2002b), *Tomato spotted wilt virus* (TSWV) NSs (Prins *et al.*, 1996) and *Cucumber mosaic virus* (CMV) 2b (Bucher *et al.*, 2003), or under the control of the Lhca3.1 promotor in case of GUS (Nap *et al.*, 1993). All binary constructs were introduced into

Agrobacterium tumefaciens strain LBA4404. Each agrobacterium culture was grown overnight at 28 °C in 2 ml LB-3 medium (1% peptone, 0.5% yeast extract, 0.4% NaCl, 0.1% KCl, 0.3% $MgSO_4$) containing 20 μg/ml of rifampicin and 50 μg/ml of kanamycin as selection markers for agrobacteria and binary constructs, respectively. The rest of the ATTA procedure (also referred to as agroinfiltration) was performed as descrived in the Chapter 3 of this dissertation. On the third day post ATTA, the leaves were additionally inoculated with the various CPMV constructs.

In vitro transcription

Capped *in vitro* transcripts of wild type RNA1 segment and of M19GFP7, M29GFP-ΔCP, M8GFP2A, M19GFP2A and M19GFP7-F8 RNA2 segments were obtained from the corresponding *in vitro* transcription plasmid constructs mentioned above using the Message Machine^{TM} kit (Ambion). Plasmid DNA templates were purified with midiprep columns (Qiagen). *In vitro* transcription reactions were carried out in 80 μl reactions using T7 RNA polymerase. Each reaction contained 4 μg of template DNA, 20 units of *Cla*I to linearise the DNA, 40 μl 2X NTP/Cap mixture (2X NTP/Cap mixture: 15 mM ATP, 15 mM CTP, 15 mM UTP, 3 mM GTP, 12 mM Cap analogue), 6 μl of enzyme mixture (T7 RNA polymerase and RNAse inhibitor) and reaction buffer in adequate final concentration. The reactions were incubated at 37 °C for 3 hours. Free nucleotides and proteins were removed by RNA precipitation with LiCl by adding, to each initial 80 μl reaction, 120 μl of water, 100 μl LiCl precipitation solution (7.5 M LiCl in 50 mM EDTA) and incubation for at least 30 min at -20 °C. Precipitated *in vitro* transcripts were then centrifuged at 4 °C for 15 min at maximum speed in an Eppendorf centrifuge; the pellet was washed with 1 ml 70% ethanol and resuspended in 80 μl water. *In vitro* transcripts were quality checked on agarose gels, quantified by spectrophotometry and kept at -20 °C until used as inoculum.

Virus inoculations

All virus inoculations were performed mechanically onto carborundum-dusted leaves at the adaxial surface. Inoculations performed onto agroinfiltrated leaves were done 3 days post ATTA.

In vitro transcript inoculations onto tobacco plants were done using a mixture of 10 μg wild-type RNA1 *in vitro* transcripts plus 10 μg *in vitro* transcripts of the different GFP-expressing RNA2 constructs in 25 μl final volume of inoculation buffer (100 mM NaH_2PO_4, 100 mM $MgCl_2$, pH 6.0, containing 20 units of RNAse inhibitor RNAsin, Gibco BRL) per leaf.

To obtain virions from GFP-expressing CPMV constructs, wild-type RNA1 *in vitro* transcripts were co-inoculated with *in vitro* transcripts of the RNA2 constructs (approximately 5 μg of each RNA) onto primary leaves of cowpea plants. Extracts from infected cowpea leaves containing the recombinant virions were then used for further inoculations onto tobacco. Although the concentration of virus in extracts from infected cowpea leaves was not known, all plants analysed in a particular experiment were inoculated with the same extract of infected cowpea leaves.

Fluorescence microscopy

Imaging of GFP fluorescence in plant tissue (i.e. from GFP-expressing CPMV or GFP-expressing agroinfiltration construct) was done with a Zeiss LSM510 laser scanning microscope. GFP fluorescence was detected by excitation with blue laser light at 488 nm and emission through a 505-530 nm bandpass filter.

Analysis of number and size of CPMV infection foci

Quantification of the number and size of CPMV infection foci was based on GFP fluorescence produced by the various CPMV constructs using a Zeiss LSM510 laser scanning microscope and fresh leaf material 3-4 dpi. At least three plants inoculated on three leaves of comparable size were used per treatment. Results from at least two repetitions were pooled and plotted in the same graph. The number of M19GFP7 or M29GFP-ΔCP infection foci was quantified per half leaf by visualizing GFP fluorescence at 10X magnification. To record the size of M8GFP2A or M19GFP7-F8 infection foci, each fluorescent spot was imaged at 10 or 25X magnification and the number of GFP-fluorescent epidermal cells per spot was quantified. M8GFP2A or M19GFP7-F8 foci were categorized as single cell, 2-5 cells (small), 6-15 cells (medium) or 16-30 cells (large). To record the size of M19GFP7 infection foci, which were considerably larger than those found with M8GFP2A and M19GFP7-F8, the fluorescent area instead of number of fluorescing cells was measured. Each M19GFP7 infection focus was imaged at 2.5 or 10X magnification and the areas were measured using the Zeiss LSM510 software and categorized as 0.10-0.49 mm^2 (small), 0.50-0.99 mm^2 (medium) to 1.00-3.00 mm^2 (large).

RESULTS

Upon RNA inoculation, potyviral HC-Pro increases the number of primary CPMV infection sites

To test whether RNA silencing plays a role in the establishment of a primary infection, *in vitro* transcripts of GFP-expressing CPMV constructs were inoculated on *N. benthamiana* plants in the absence or presence of a strong heterologous suppressor of RNA silencing, i.e. potyviral HC-Pro (Anandalakshmi *et al.*, 1998; Kasschau & Carrington, 1998). HC-Pro of CABMV was provided in two ways, either by using HC-Pro transgenic *N. benthamiana* or by ATTA performed into the abaxial part of the leaves. The transgenic *N. benthamiana* plants expressed HC-Pro constitutively under the control of the 35S promoter (Mlotshwa *et al*., 2002b). Since viral *in vitro* transcripts were inoculated onto the adaxial surface of the leaf, it was essential to check whether the upper epidermis layer would efficiently be transformed by ATTA. To verify this, *N. benthamiana* plants were agroinfiltrated with a GFP construct to visualise transformed cells. Both the abaxial and adaxial epidermal cells of the infiltrated leaf areas were transformed and expressed the GFP reporter gene. Cells of the mesophyll tissue and minor veins, but not the major veins were also transformed efficiently (Fig. 1a, 1b and 1c). Furthermore, *N. benthamiana* plants agroinfiltrated with HC-Pro or with GUS reporter gene constructs expressed the proteins at 3 and 5 days post ATTA, as detected by Western blotting and GUS staining assay respectively (data not shown). These results demonstrate that ATTA is an efficient tool to deliver HC-Pro as heterologous silencing suppressor.

For easy monitoring of virus infection foci, leaves were inoculated with in vitro

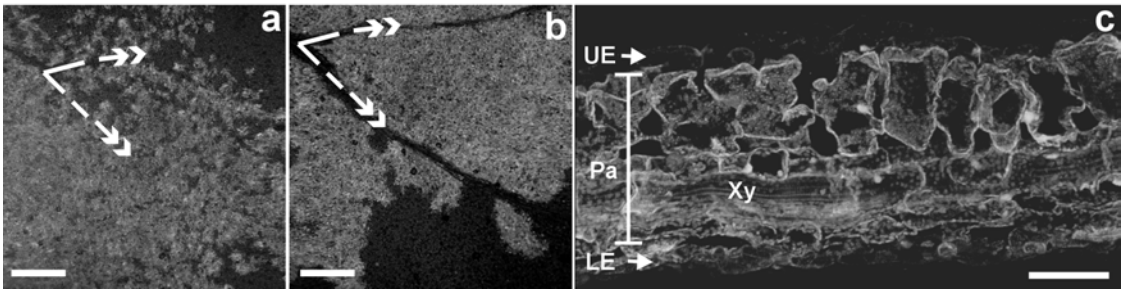

Figure 1. Efficiency of agroinfiltration of a GFP construct in *N. benthamiana* leaves. (a) GFP-fluorescence at the abaxial leaf surface 3 days post agroinfiltration. Arrows indicate the position of major veins. (b) GFP-fluorescence at the adaxial surface of the same leaf area in (a), and absence of fluorescence in the major veins (arrows). (c) GFP fluorescence in cells of a representative cross-section through an infiltrated leaf area shows GFP construct efficiently agroinfiltrated through all cell layers within leaf lamina, including most of the upper epidermis (UE) cells. Lower epidermis (LE). Parenchyma (Pa). Xy, autofluorescence of xylem. Magnification bars indicate 1 mm in (a) and (b), and 100 μm in (c).

transcripts of a GFP-expressing CP deletion mutant of CPMV (M29GFP-ΔCP; see Fig. 2a). This mutant does not form virions and consequently infections remain restricted to single cells (Verver et al. 1998 and Fig. 2b). The expression of GFP by the mutant virus allowed easy quantification of primary infection foci. Three to four dpi (i.e. 6-7 days post ATTA respectively), the number of infection foci was counted and the results of two repetitions per treatment were averaged. In HC-Pro transgenic plants inoculated with M29GFP-ΔCP, the average number of infection foci increased 68 fold compared to non-transgenic plants (Fig. 3a). This positive effect on the establishment of primary infection was also observed when HC-Pro was provided through ATTA, albeit that the increase was 5 fold compared to control plants transformed with the GUS gene (Fig. 3a). To verify whether the stimulatory effect of HC-Pro was caused by its RNA silencing suppressor activity, another strong viral suppressor TSWV NSs (Bucher et al., 2003) was tested. The effect of this protein was only tested by ATTA, as no NSs transgenic plants were available. Expression of NSs was verified by Western blotting at 3 and 5 days post ATTA (data not shown). Like with HC-Pro, the number of primary infection foci increased 4 fold in the presence of NSs delivered by ATTA (Fig. 3a). However, no effect was found with the 2b protein, the suppressor of silencing of CMV (Fig. 3a).

To exclude that the effect of HC-Pro on primary infection efficiency was due to the crippled nature of the mutant M29GFP-ΔCP, CPMV recombinant M19GFP7 was tested. M19GFP7 encodes all viral proteins, and additionally it expresses GFP as free protein (Fig. 2a). Moreover this construct infects plants locally and systemically at wild type speed (Gopinath *et al*., 2000; Silva *et al*., 2002). When provided as in vitro transcript the number of successful infection foci of M19GFP7 in HC-Pro transgenic plants was 14 fold higher than in non-transgenic control plants (as quantified 3 dpi, Fig. 3b), indicating that the stimulatory effect of HC-Pro was not dependent on the mutant genotype.

The fact that HC-Pro increased the number of primary infection sites upon CPMV RNA inoculation (i.e. in vitro transcripts) indicates that CPMV is confronted with RNA silencing already in the primary infected cells.

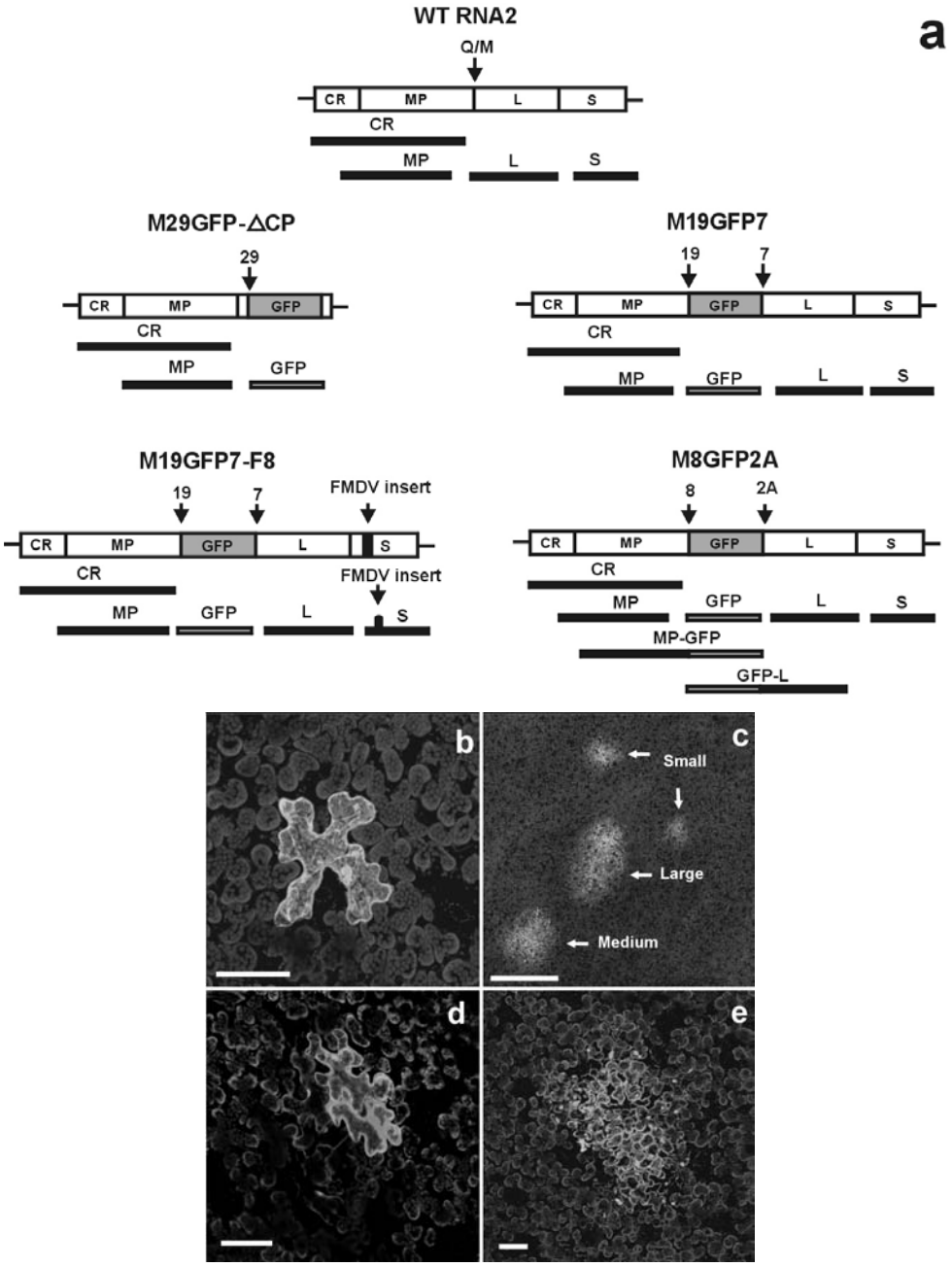

Figure 2. CPMV RNA-2 constructs and their performance *in planta*. (a) Schematic representation of CPMV wild type (WT) RNA2 and GFP-expressing RNA2 constructs M19GFP7, M29GFP-ΔCP, M19GFP7-F8 and M8GFP2A. The GFP gene was inserted between the movement protein (MP) and the large coat protein (L) coding regions in an *in vitro* transcription vector. The constructs M19GFP7 and M8GFP2A encode for GFP and all RNA2 proteins. Construct M29GFP-ΔCP lacks the coat proteins coding region and M19GFP7-F8 contains a 10 amino acids insert of the FMDV VP1 protein in the small coat protein (S) coding region. The arrows flanking the GFP gene indicate proteolytic cleavage sites. Q/M corresponds to a proteolytic cleavage found in WT RNA2. Cleavage sites 19, 29 and 8 were created by respectively duplicating 19, 29 or 8 amino acids including the Q/M site from the N terminus of L. Cleavage site 7 was created by duplicating 7 amino acids including the Q/M site from the C terminus of MP. Cleavage site 2A corresponds to the 2A catalytic peptide from FMDV. Due to the partial cleavage of sites 2A and 8, M8GFP2A express free L protein, MP and GFP, but also GFP-L and MP-GFP fusion proteins. Cleavage sites 19 and 7 in M19GFP7 and M19GFP7-F8 generate free MP, GFP, L and S. Cleavage site 29 in M29GFP-ΔCP generates free MP and GFP. (b) M29GFP-ΔCP single cell restricted infection 4 dpi. (c) M19GFP7 typical small, medium and large infection foci at 3 dpi. (d) Typical M19GFP7-F8 small infection foci 3 dpi. (e) Typical M8GFP2A large infection foci 3 dpi. CR, cofactor for RNA2 replication. Bars indicate 100 μm in (b), (d) and (e), and 1 mm in (c).

Upon virion inoculation, potyviral HC-Pro does not increase the number of primary CPMV infection sites

To investigate whether HC-Pro has a similar incremental effect on the number of primary infection sites when inoculating CPMV virions instead of RNA, plants were inoculated with purified virions of recombinant M19GFP7 in the presence or absence of HC-Pro. The number of infection foci in non-transgenic and HC-Pro transgenic plants was recorded 3 dpi. Remarkably, the number of foci was not higher in HC-Pro transgenic plants (Fig. 3c). The difference between inoculations with M19GFP7 virions (Fig. 3c) and in vitro transcripts (Fig. 3b) is the presence of the CPs when virions are used, whereas in vitro transcripts only express the CPs upon translation of the inoculated viral RNA. These observations suggest that HC-Pro, a suppressor of RNA silencing, confers a protection to CPMV RNA that is equally effective for the establishment of primary infections as the CPMV coat protein(s).

Heterologous suppressors of RNA silencing do not enhance local spread of CPMV

The previous experiments suggest that RNA silencing plays an inhibitory role in initiation of virus infection. Subsequently, we investigated whether RNA silencing had a similar effect on the progress of infection from a primary infected cell, i.e. local spread of infection. To assess the influence of RNA silencing on CPMV local spread, we measured the area of infection foci in transgenic HC-Pro plants and non-transgenic plants inoculated with M19GFP7 virions or *in vitro* transcripts. Infection foci were categorised as small, medium or large, based on measurements of the area of infected epidermal cells (Fig. 2c). At 3 dpi, the size of M19GFP7 infection foci was similar in leaves of both plants inoculated with virions (Fig. 4a) or RNA (Fig. 4b). The fact that HC-Pro did not enhance the local spread of CPMV indicated that RNA silencing did not noticeably affect the viral local spread.

However, a possible explanation for finding no apparent effect of RNA silencing opposed to CPMV local spread could be that M19GFP7 replicated and moved fast enough to escape these counteracting effects by, for instance, spreading ahead of siRNA. We considered the premise that slowing down the speed of CPMV local spread would give the infected plant time to react against the virus by a RNA silencing mediated mechanism. CPMV constructs M19GFP7-F8 and M8GFP2A, which both spread slower than wild type virus were used to test this hypothesis. M19GFP7-F8 is a M19GFP7 based recombinant that expresses a 10 amino acid sequence from *Foot and mouth disease virus* (FMDV) VP1 coat protein and free GFP (Fig. 2a and see Porta *et al.*, 2003). The M19GFP7-F8 recombinant spreads to a limited extent in inoculated *N. benthamiana* leaves (Fig. 2d), but does not infect the plants systemically.

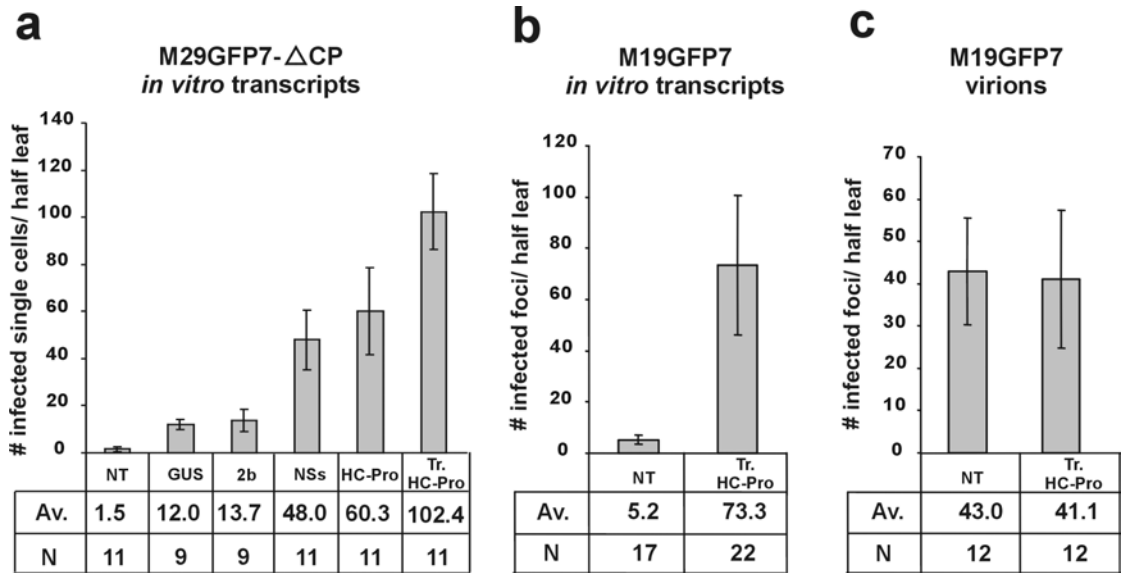

Figure 3. Effect of heterologous suppressors of RNA silencing on the number of primary infection foci. (a) Average (Av.) number of infected single epidermal cells per half leaf of non-transgenic (NT) and HC-Pro transgenic (Tr. HC-Pro) *N. benthamiana* plants, and of plants agroinfiltrated with GUS, 2b, NSs or HC-Pro construct, inoculated with M29GFP-ΔCP *in vitro* transcripts. (b, c) Average number of infection foci per half leaf in NT or Tr. HCPro leaves inoculated with (b) M19GFP7 *in vitro* transcripts or (c) M19GFP7 virions. N, Number of half leaves observed per treatment.

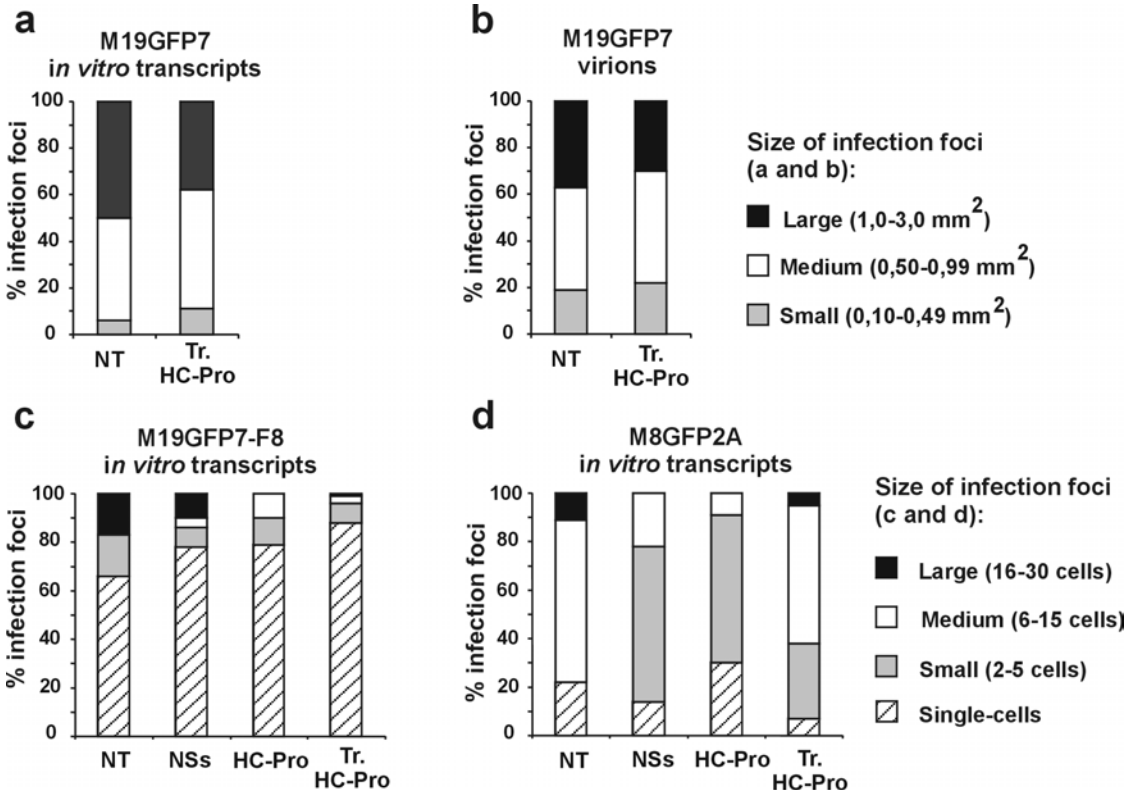

Figure 4. Effect of heterologous suppressors of RNA silencing on the size of infection foci. (a, b) Percentage of small, medium and large infection foci in leaves of NT or Tr. HCPro plants inoculated with (a) M19GFP7 *in vitro* transcripts or (b) M19GFP7 virions. The size of the foci corresponds to the area of infected epidermal cells within each focus. (c, d) Percentage of single-cells, small, medium and large infection foci in leaves of NT and Tr. HC-Pro plants, and of leaves agroinfiltrated with NSs or HC-Pro construct, inoculated with (c) M19GFP7-F8 or (d) M8GFP2A *in vitro* transcripts. The size of infection foci corresponds to the number of infected epidermal cells within each focus.

M8GFP2A is a CPMV recombinant that encodes GFP between the MP and L coat protein, flanked by two cleavage sites that are partially processed, thus producing free MP, free GFP, free L as well as MP-GFP and GFP-L fusion proteins upon infection (Fig. 2a and see Gopinath *et al.*, 2000). Like M19GFP7-F8, this recombinant exhibits limited spread in inoculated *N. benthamiana* leaves (up to 30 epidermal cells, Fig. 2e) and is defective in systemic spread.

Both M19GFP7-F8 and M8GFP2A move slower than M19GFP7 (wild type speed of spread) since, at similar time points post inoculation, the M19GFP7 foci are always larger than M19GFP7-F8 or M8GFP2A. For instance, in non-transgenic plants at 3 dpi M19GFP7 foci are up to 3 mm^2 in area, whereas M19GFP7-F8 and M8GFP2A spread to a maximum of 0.3 mm^2, corresponding to approximately 30 epidermal cells (compare Fig. 4b, 4c and 4d). Also at 2 dpi M19GFP7 foci are larger than those of M19GFP7-F8 and M8GFP2A (data not shown).

To assess whether RNA silencing influences the local spread of CPMV both slow moving recombinants were tested in the presence of suppressors HC-Pro and NSs. *N. benthamiana* plants expressing HC-Pro (by agroinfiltration or transgenically) or NSs (by agroinfiltration) were inoculated with *in vitro* transcripts of M19GFP7-F8 or M8GFP2A. At 3 dpi, the size of infection foci of both recombinants was similar in the presence or absence of either suppressors of silencing (Fig. 4c and 4d). These results indicate that even when CPMV local spread was slow, RNA silencing did not considerably affect the progress of infection.

DISCUSSION

In the presented experiments the influence of suppressors of RNA silencing on the early stages of CPMV infection was investigated. It was demonstrated that strong heterologous suppressors, the potyviral HC-Pro and tospoviral NSs, significantly increased the number of CPMV infection foci in leaves inoculated with CPMV *in vitro* transcripts. However, no effect was observed with the cucumoviral 2b suppressor. These observations suggest that RNA silencing plays a role in early defence against plant virus infection, already in the initially infected cells. The different effects observed with suppressors HC-Pro and NSs on one hand and 2b on the other can be explained by the difference in mode of action of these proteins. The 2b protein suppresses a systemic signalling step in the silencing pathway (Brigneti *et al*., 1998), while HC-Pro and NSs operate more immediate by suppressing directly a maintenance step of RNA silencing which results in prolongation of viral RNA replication (Kasschau *et al*., 1997; Pruss *et al*., 1997). Both HC-Pro and NSs have been shown to inhibit the accumulation of siRNA through an unknown mechanism (Llave *et al*.,

2000; Mallory *et al*. 2001; Bucher *et al*., 2003). Furthermore, Mlotshwa *et al*. (2002c) showed that in protoplasts infected with CPMV-HC-Pro, a recombinant virus that also produces HC-Pro, the latter localised to cytoplasmic patches known to contain the CPMV replicative dsRNA (the putative trigger of RNA silencing) (Carette *et al.*, 2002b), although during potyviral infection, HC-Pro is distributed over the whole cytoplasm.

Nevertheless, it cannot be excluded that HC-Pro as well as NSs protect CPMV RNA against non-specific degradation by cellular RNases, rather than protection against the RNA silencing machinery. HC-Pro is capable of binding ssRNA *in vitro* in a sequence non-specific manner (Maia & Bernardi, 1996), but this property is most probably related to its role in cell-to-cell movement rather than protection of RNA against non-specific degradation.

Although HC-Pro and NSs increased the number of CPMV infection foci in leaves inoculated with in vitro transcripts, no effect of these suppressors was found upon inoculation with virions. This could be partially due to protection of the viral RNA by the CPs against non-specific degradation by nucleases. Moreover it could also reflect suppression of RNA silencing by the CPs, in accordance to recent findings that the small CP (S) of CPMV is a suppressor of RNA silencing (Liu *et al*., in press).

To date several viral suppressors of RNA silencing have been identified (Brigneti *et al.*, 1998; Voinnet *et al.*, 1999; Voinnet *et al.*, 2000; Yelina *et al.*, 2002; Pfeffer *et al.*, 2002; Dunoyer *et al.*, 2002; Bucher *et al.*, 2003), all of which are viral non-structural proteins, with the exception of the CP (P38) from *Turnip crinkle carmovirus* (TCV) (Thomas *et al.*, 2003). The P38 protein was shown to be a very strong suppressor of RNA silencing in agroinfiltration assays, but not when presented in the virion context, upon inoculation with virus particles. The N-terminal sequence of P38 appears to be important for this activity and forms part of an unexposed R-domain that interacts with the viral RNA within the virions. It was speculated that TCV in this way could regulate the suppression activity of P38, i.e. no activity when P38 is present in virion conformation. In the case of CPMV S coat protein, the regulation of RNA silencing suppression activity could equally be done by a conformational presentation. S in either a virion or non-virion manner are possible forms of CPMV regulation of S suppression of RNA silencing activity.

CPMV S protein combines functions in encapsidation and suppression of RNA silencing which is a structural protein immediately available upon virus transmission by beetle vectors. If RNA silencing already interferes with the initiation of virus infection, delivery of virions together with a suppressor of silencing, as is evident with the beetle transmitted CPMV, may constitute a major biological advantage. Also in the case of potyviral transmission by aphids, HC-Pro is delivered to the plant cell together

with the virions. Besides being a suppressor of silencing, HC-Pro is also an essential helper factor involved in transmission of the virus by the insect vector aphid (Thornbury et al., 1985). Also in the case of TSWV NSs, a virus naturally transmitted by thrips, large amounts of this suppressor protein are found in the salivary glands together with virions (Wijkamp et al., 1993) and virions and NSs are probably injected simultaneously into the plant cell.

In experiments using a CPMV recombinant that spreads in wild type fashion (i.e. M19GFP7), no evidence was found that RNA silencing represents a noticeable barrier against CPMV local spread. Therefore, we considered the hypothesis that slowing down the local spread of CPMV could eventually result in arrest viral infection due to spread of siRNAs ahead of the CPMV spread/infection front. Nevertheless, the local spread of CPMV recombinants, which are slower than wild type virus (i.e. M19GFP7-F8 and M8GFP2A), did not improve in the presence or absence of HC-Pro or NSs. In conclusion, CPMV must have developed a time-independent strategy to escape putative RNA silencing effects during local spread, by for instance preventing the formation of siRNAs.

Intriguingly, M19GFP7-F8 and M8GFP2A must be limited in local spread for a reason other then RNA silencing hindrance. Besides RNA silencing, plants may rely also on innate immune responses to combat pathogens. Innate immune response involves, for instance, recognition of potential pathogens by a resistance gene (R) (Flor, 1971) that initiates a battery of active defence responses (Dangl & Jones, 2001). Nevertheless, the so-called basal resistance mechanism can limit the growth of a virulent pathogen even in the absence of R function (Feys & Parker, 2000). It is possible that basal resistance is the mechanism hampering the local spread of M19GFP7-F8 and M8GFP2A constructs in *N. benthamiana* plants, where R-mediated resistance against CPMV is definitely not present.

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CHAPTER 5

Dissecting the *Cowpea mosaic virus* systemic infection process using the semi-permissive plant host *Nicotiana tabacum*

SUMMARY

Cowpea mosaic virus (CPMV) spreads locally between cells as virions, utilizing plasmodesma-penetrating tubular structures build-up from the viral encoded movement protein. The vascular movement of CPMV does not seem to be tubule-guided, but the exact mechanisms involved in this process are not well understood. Here we have investigated the usefulness of a semi-permissive host, i.e. *N. tabacum,* to further unravel the systemic infection process of CPMV. CPMV does not infect *N. tabacum* systemically despite extensive local spread in inoculated leaves. It is shown here that neither incubation temperature nor RNA silencing-, salicylic acid- or ethylene-mediated resistance mechanisms are the limiting factor in CPMV systemic infection. Although CPMV-infected *N. tabacum* plants are normally asymptomatic, symptoms (i.e. necrotic lesions) within the inoculated leaves were observed at low temperature. Grafting experiments indicated that CPMV is not capable of phloem loading in *N. tabacum*, a step that normally follows cell-to-cell movement. These findings make *N. tabacum* an interesting system for investigations of the host factors involved in CPMV vascular movement.

INTRODUCTION

The systemic infection of a host plant by viruses depends on the ability of the virus to replicate in the initially infected cell, to spread locally within the inoculated leaf (cell-to-cell movement), and to move long-distance through the vascular tissue (vascular movement), by entering the phloem stream (loading) for viral transport to sink tissues (i.e. roots, young leaves, flower buds and fruits), where it exits the phloem (unloading) and re-establishes infection.

In the case of *Cowpea mosaic virus* (CPMV), different plant species present different virus infection patterns. For instance, *Vigna unguiculata* (cowpea) is a permissive host for CPMV, i.e. it supports all steps of viral systemic infection. Also *Nicotiana benthamiana* is a permissive host for CPMV, whereas *Nicotiana tabacum* is a semi-permissive host, which confers some impediment of unknown nature against CPMV systemic infection (Huber *et al.*, 1977; Nida *et al.*, 1992).

CPMV is a bipartite ssRNA virus with RNA1 encoding the proteins involved in viral replication and RNA2 encoding the movement protein (MP), the small (S) and large (L) coat proteins (CPs) (reviewed by Pouwels *et al.*, 2002a). During cell-to-cell movement, a tubule build of MP molecules replaces the desmotubule within the plasmodesma and mediates transport of whole virions into neighbouring cells (Wellink & van Kammen, 1989; van Lent *et al.*, 1990; Kasteel *et al.*, 1993), until the virus reaches the phloem. Within the phloem tissue, the loading of CPMV into the sieve elements does not seem to be tubule-guided (Silva *et al.*, 2002) and probably requires the CPs (Chapter 3 of this thesis), but further aspects of CPMV vascular movement are not yet understood. Here we have studied whether the semi-permissive *N. tabacum* host is a useful tool for further dissecting the systemic infection process of CPMV.

Although inoculated leaves of *N. tabacum* support replication of CPMV (Huber *et al.*, 1977; Nida *et al.*, 1992), it remained unknown how extensive CPMV cell-to-cell movement is in this host and whether virus loading into the phloem actually occurs in these plants. Therefore the extent of CPMV cell-to-cell movement and phloem loading in *N. tabacum* plants was studied, as well as the potential presence of a systemic resistance signal travelling ahead of infection and preventing viral systemic spread. Furthermore, we tested the hypothesis that *N. tabacum* plants could employ RNA silencing (for recent review: Yu & Kumar, 2003) as an active resistance mechanism to prevent CPMV systemic infection. Moreover, the effect of incubation temperature on CPMV systemic spread in *N. tabacum* was evaluated. Finally, the involvement of the salicylic acid- (SA) and ethylene-induced resistances, active in numerous plant-pathogen combinations (reviewed by Dong, 1998), was also investigated in the *N. tabacum*-CPMV system.

MATERIALS AND METHODS

Plants

Nicotiana tabacum cv. Samsun was used in most of the experiments, unless otherwise stated. Transgenic *N. tabacum* cv. Xanthi nc line NahG-10 (Gaffney *et al*., 1993), expressing the *Pseudomonas putida* strain PpG7 gene *nahG* encoding salicylate hydroxylase, was a gift from Dr. Leslie Friedrich, Syngenta Biotechnology, North Carolina-US. Transgenic *N. tabacum* cv. Samsun NN line SH-L(4) (Bi *et al*., 1995), expressing the *P. putida* strain NC1B9819 salicylate hydroxylase gene homologous to *nahG*, was a gift from Dr. Robert Darby, Institute of Biological Sciences, University of Wales, Aberystwysth-UK. Transgenic *N. tabacum* cv. Samsun NN line Tetr-18 (Knoester *et al*., 1998), which expresses the gene *etr1-1* (mutant of the gene *etr1*) conferring insensitivity to ethylene, was a gift from Dr. Huub Linthorst, Institute of Biology, Leiden University, Leiden-The Netherlands. *Nicotiana benthamiana* plants were used to maintain some virus inocula, as control in some experiments and as scions in grafts. The tobacco seeds were sown in sterilised soil and grown in a growth chamber at 23 °C with 16 hours light. *Vigna unguiculata* cv. California Blackeye (cowpea) plants were used to obtain viral inocula and for back inoculation assays. Cowpea plants were grown under the same conditions as described for the tobacco plants.

Viruses

Wild type CPMV (CPMV-WT) inoculum was maintained in cowpea plants, whereas wild type Tomato spotted wilt virus (TSWV), Potato virus Y (PVY) and Cucumber mosaic virus (CMV) inocula were maintained in *N. benthamiana* plants. GFP-expressing CPMV (CPMV-GFP) was produced using a combination of T7-promoter-driven in vitro transcription constructs of wild type RNA1 and a GFP-expressing RNA2. The RNA1 construct has been described previously by Eggen *et al*., (1989), and the RNA2 construct M19GFP7 was described by Gopinath *et al*., (2000). Co-inoculation of in vitro transcripts of RNA1 and M19GFP7 result in CPMV-GFP virus, which expresses all viral proteins as in CPMV-WT plus free GFP molecules. Extracts from CPMV-GFP infected cowpea leaves were then used to further amplify the inoculum in cowpea. CPMV-WT or CPMV-GFP virions were purified from infected cowpea leaves as described by van Kammen (1967) except for the sucrose gradient step that was omitted.

Agrobacterium T-DNA transient-expression assay (ATTA)

The constructs used for ATTA (also referred to as agroinfiltration) that encode for suppressors of RNA silencing, *Cowpea aphid borne mosaic virus* (CABMV) HC-Pro (Mlotshwa *et al.*, 2002b) and CMV-2b (Bucher *et al.*, 2003), were available in the binary vector pBIN19 and express the proteins under the control of the 35S promoter. The binary constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404. Each agrobacterium culture was grown overnight at 28 °C in 2 mL LB-3 medium (1% peptone, 0.5% yeast extract, 0.4% NaCl, 0.1% KCl, 0.3% $MgSO_4$) containing 20 μg/ml of rifampicin and 50 μg/ml of kanamycin as selection markers for agrobateria and binary constructs, respectively. The rest of the ATTA procedure was performed as described in Chapter 3 of this dissertation.

Virus inoculations

Tobacco plants (*N. benthamiana* or *N. tabacum*) had about four leaves (i.e. around one month-old) when inoculated, unless stated otherwise. Cowpea plants were inoculated 8-9 days post sowing. *In vitro* transcripts of RNA1 and M19GFP7 were co-inoculated onto cowpea leaves as described by Silva *et al.*, 2002. Mechanical inoculations of agroinfiltrated *N. tabacum* leaves were performed 2 days post ATTA onto the carborundum-dusted adaxial surface. Co-inoculations of CPMV-GFP with either PVY, TSWV or CMV were performed by first inoculating leaves with purified CPMV-GFP, immediately followed by inoculation with extracts of plants infected with PVY, TSWV or CMV. In general, *N. tabacum* leaves were inoculated with 100-200 μl of a 1 mg/ml purified CPMV-WT or CPMV-GFP suspension in PBS. For back inoculation assays, extracts of individual tobacco leaves or roots of individual tobacco plants were inoculated onto two primary leaves of a single cowpea plant. Inoculated plants were incubated in growth chamber at 23 °C with 16 hours light, unless stated otherwise.

Grafts

Six to seven weeks old *N. benthamiana* (scions) and *N. tabacum* cv. Samsun (rootstocks) plants were used to make grafts. The graft union, which was sealed with tape, was cut as a straight incision into the rootstock stem and as a V-shaped tip at the scion stem end. After grafting, both scion and rootstock had 2-3 leaves left. The scions (including the graft union) were immediately covered with transparent plastic bags containing a wet piece of cotton to prevent tissue desiccation. The grafts were then

incubated in a growth chamber at 23 °C with 16 hours light. At 6 days post grafting, the plastic bags were removed and virus inoculations were performed onto all rootstock leaves available. The presence of virus in both rootstocks and scions was investigated at 9 dpi by back inoculation of leaf extracts onto cowpea plants.

Fluorescence microscopy and imaging

The CPMV-GFP fluorescence was monitored/imaged with a Leica stereo fluorescent system consisting of a Wild M3Z stereo microscope equipped with UV illumination and a GFP-plus filter set (excitation 480/40 nm; dichroic beam splitter 505 nm LP; barrier filter 510 nm LP). Images of whole leaves was done by using a digital camera (Richo), ISO 400, 4 seconds exposure with a wratten gelatine filter No. 58 (Kodak- cat. 1495860) for GFP fluorescence imaging under UV illumination, or no filter for bright field imaging. More detailed imaging of infected areas was done by using a Zeiss LSM510 laser-scanning microscope. GFP fluorescence was observed through excitation with blue laser light at 488 nm and emission through a 505-530 nm bandpass filter.

RESULTS

CPMV does not infect *N. tabacum* systemically despite extensive local spread in inoculated leaves

In *N. tabacum* plants CPMV systemic infection is hampered by a so far unknown mechanism. To investigate the limiting step(s) of CPMV systemic infection in *N. tabacum* plants CPMV-GFP was used to facilitate visualization of viral infection *in locu*. Initially, extracts of CPMV-GFP infected cowpea leaves were used to inoculate different cultivars of *N. tabacum* (i.e. cv. Samsun, cv. Samsun NN, cv. Xanthi, cv. White Burley and cv. Burley 21) but no fluorescent infection foci were ever observed, while *N. benthamiana* became readily infected upon the same inoculum pressure (data not shown). As it was suspected that the inoculum pressure of cowpea leaf extracts was not high enough to result in successful infection of *N. tabacum*, the virus titre was increased by using purified CPMV-WT or CPMV-GFP at a concentration of 1 mg/ml as inoculum. CPMV-WT and CPMV-GFP systemic infection of *N. tabacum* cv. Samsun, cv. Samsun NN and cv. Xanthi nc was followed in a time course of 21 days. CPMV infection was never observed in the non-inoculated (i.e. upper) leaves (Table 1), although the inoculated (i.e. lower) leaves were infected until 21 dpi (data not shown).

Table 1. Time course of CPMV systemic infection in *N. tabacum* non-inoculated upper leaves

	Nr. of systemically infected plants [(b)]/Nr. of inoculated plants					
Plant-virus[(a)]	**4 dpi**	**7 dpi**	**10 dpi**	**14 dpi**	**17 dpi**	**21 dpi**
***N. tabacum* 'Samsun'**						
CPMV-WT	0/2	0/2	0/2	0/2	0/2	0/2
CPMV-GFP	0/2	0/2	0/2	0/2	0/2	0/3
***N. tabacum* 'Samsun NN'**						
CPMV-GFP	0/2	0/2	0/2	0/2	0/2	0/2
***N. tabacum* 'Xanthi nc'**						
CPMV-GFP	0/2	0/2	0/2	0/2	0/2	0/2
Nr of leaves[(c)]	2-3	3-4	3-5	6-8	7-10	10-12

(a) Inoculations were done with purified CPMV-WT or CPMV-GFP virions, onto 1 lower leaf/plant.
(b) Systemic infection of non-inoculated upper leaves was checked by GFP fluorescence and back inoculation of extracts of each upper leaf on a cowpea plant.
(c) Number of upper leaves additional to the inoculated lower leaves.

Since roots are strong sink tissues and usually become systemically infected even before the sink leaves (Silva *et al.*, 2002), we also checked CPMV-GFP or CPMV-WT infection in *N. tabacum* roots in a time course, using *N. benthamiana* as positive control. GFP fluorescence was not useful for detection of CPMV-GFP in roots because roots of non-infected plants display green autofluorescence (data not shown). Presence of CPMV-WT or CPMV-GFP in plant roots was therefore monitored by back inoculation of root extracts onto cowpea plants. In roots of *N. tabacum* no CPMV infection could be found, while roots of *N. benthamiana* did become infected (Table 2). For the latter back inoculations extracts of roots from *N. benthamiana* were mixed with extracts of roots from non-infected *N. tabacum*, demonstrating that in roots of *N. tabacum* there was no substance inhibiting CPMV infection upon back inoculation with root extracts. These results confirm that CPMV is not able to establish systemic infection in the *N. tabacum* plant species.

Table 2. Time course of CPMV infection of *N. tabacum* and *N. benthamiana* roots

Root collection	Nr. of plants with infected roots[(b)] per		
	Nr. of CPMV-WT inoculated *N. tabacum*[(a)]	**Nr. of CPMV-GFP inoculated** *N. tabacum*[(a)]	**Nr. of CPMV-WT inoculated** *N. benthamiana*[(a)]
1 dpi	0/4	0/4	0/3
2 dpi	0/4	0/4	0/3
3 dpi	0/4	0/4	0/3
4 dpi	0/4	0/4	3/3
7 dpi	0/4	0/4	3/3

(a) *N. tabacum* cv. Samsun plants were inoculated with purified CPMV-WT or CPMV-GFP virions, onto 1 leaf/plant. *N. benthamiana* plants were inoculated with leaf extracts of CPMV-WT infected cowpea, onto 3 leaves/plant.
(b) Root extracts were back inoculated on cowpea plants.

To investigate whether limited cell-to-cell movement would be the reason why CPMV is unable to infect *N. tabacum* systemically, *N. tabacum* leaves were inoculated with purified CPMV-GFP virions and were inspected for local spread of the virus. The CPMV-GFP inoculated leaves displayed no apparent symptoms at 4 dpi, but local virus spread could be readily followed by visualisation of the GFP fluorescence signal with the naked eye (Fig. 1a). At 7 dpi, plants were still asymptomatic and the intensity of CPMV-GFP fluorescence had decreased (Fig. 1a). At later time points, i.e. from 10 dpi on, *N. tabacum* plants remained symptomless and CPMV-GFP fluorescence was no longer visible with the naked eye (Fig. 1a) but only detectable at higher magnification in the confocal microscope (Fig. 1f). At 21 dpi, CPMV-GFP fluorescent foci were rarely found even in the confocal microscopy (Fig. 1g). In contrast to infection of cowpea, the center of CPMV-GFP infection foci on *N. tabacum* leaves was usually less fluorescent than the edge (Fig. 1d, 1f and 1g) similar to infection in *N. benthamiana.* Interestingly, in *N. benthamiana* plants CPMV-GFP fluorescence did not fade after 10 dpi, while these leaves also developed chlorotic lesions within this time span (Fig. 1c). In cowpea, similar inoculum pressure (1 mg/ml) caused chlorotic lesions to appear as early as 4 dpi and was lethal to the inoculated leaves within 10 dpi (Fig. 1b). In cross sections of CPMV-GFP infected *N. tabacum* leaves, the virus was observed in upper epidermis, mesophyll cells and lower epidermis, infecting the leaf lamina even in the vicinity of the vascular bundles (Fig. 1e). Whatever the barrier imposed by *N. tabacum* against CPMV systemic infection, it does not obstruct viral spread within inoculated leaves.

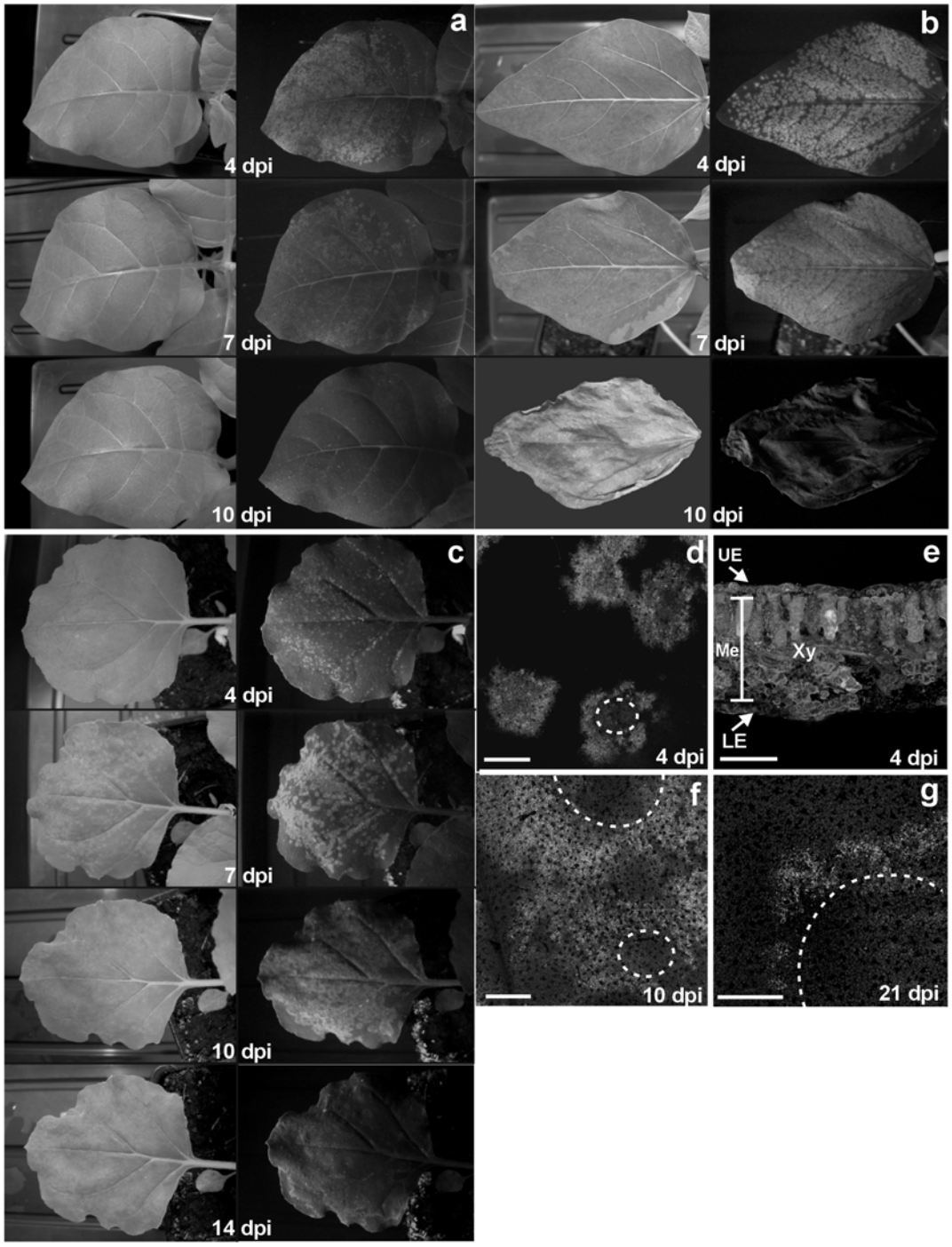

Figure 1. CPMV local spread in permissive (*N. benthamiana* and cowpea) and semi-permissive (*N. tabacum*) host plants. (a), (d), (e), (f) and (g) *N. tabacum*, (b) cowpea or (c) *N. benthamiana* leaves inoculated with purified CPMV-GFP. In (a), (b) and (c), on left side there are bright field images and on right side there are fluorescence images of corresponding infected leaves. Images (d) to (g) are confocal microscopy images. Dotted circles indicate the core of infection foci, where GFP fluorescence is less intense. Note in (e) that cross section through leaf displays extensive local spread of CPMV-GFP throughout leaf lamina. UE, upper epidermis. Me, mesophyll cells. LE, lower epidermis. Xy, autofluorescence from xylem elements indicate position of a vein within the leaf lamina. Bars represent 200 μm in (e) and 1 mm in (d), (f) and (g).

No systemic resistance signal prevents CPMV systemic infection of *N. tabacum*

To test whether *N. tabacum* plants produce a systemic signal that could block CPMV systemic spread, *N. tabacum* upper leaves were infected with CPMV in a time course from 3 until 14 days post infection of two lower leaves of these plants (Table 3). *N. tabacum* lower leaves were inoculated with purified CPMV-GFP virions or mock inoculated with PBS buffer. At all time points evaluated CPMV-GFP infected *N. tabacum* upper leaves to the same extent in plants whose lower leaves were CPMV-GFP-inoculated as in plants whose lower leaves were mock-inoculated (Table 3). This indicates that there is no apparent systemic resistance signal impeding CPMV infection in the upper leaves.

Table 3. CPMV infection of *N. tabacum* upper leaves at different dpi of inoculated lower leaves.

Inoculation of upper leaves[(b)]	**Nr. of upper leaves/plant**	**Nr. of plants with infected upper leaves[(c)] per**	
		Nr. of plants CPMV-GFP inoculated on lower leaves	**Nr. of plants mock inoculated on lower leaves**
3 dpi of lower leaves[(a)]	2	3/3	2/2
5 dpi of lower leaves	3	3/3	2/2
7 dpi of lower leaves	4	3/3	2/2
10 dpi of lower leaves	5	3/3	2/2
14 dpi of lower leaves	6	3/3	2/2

(a) Inoculation of 2 lower leaves/ plant (*N. tabacum* cv. Samsun) was done with purified CPMV-GFP virions. Based on GFP fluorescence, all inoculated lower leaves were infected at 4 dpi.
(b) All the upper leaves of each plant were inoculated with purified CPMV-GFP virions.
(c) The extent of infection of upper leaves was checked based on GFP fluorescence at 4 dpi of the upper leaves themselves.

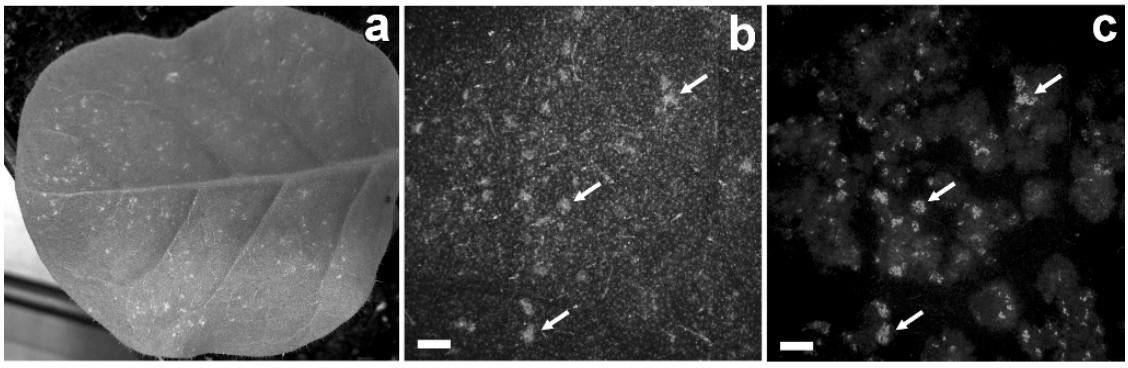

Figure 2. CPMV local infection in *N. tabacum* cv. Samsun at 15 °C. (a, b) Bright field and (c) fluorescence image of a CPMV-GFP inoculated leaf at 10 dpi. (b) and (c) correspond to the same area and depict magnified images of lesions from the leaf in (a). Arrows indicate corresponding necrotic lesions in (b) and (c). Note that necrotic lesions are associated exclusively with the fluorescent infected areas. Bars in (b) and (c) represent 1 mm.

Table 4. Effect of incubation temperature, suppression of RNA silencing, lack of accumulation of salicylic acid or ethylene-insensitivity on CPMV systemic infection of *N. tabacum* plants

	Treatment[(a)]	Effect of	Nr. of systemically infected plants/ Nr. of inoculated plants[(b)]					
			4 dpi	7 dpi	10 dpi	14 dpi	17 dpi	21 dpi
1	*N. tabacum* 'Samsun' + CPMV-GFP at 32 °C	High temperature	0/2	0/2	0/2	0/2	N	N
2	*N. tabacum* 'Samsun' + CPMV-GFP at 15 °C	Low temperature	0/2	0/2	0/2	0/2	0/2	0/2
3	*N. tabacum* 'Samsun' ATTA CABMV HC-Pro + CPMV-GFP	Suppression of silencing by potyviral HC-Pro	0/2	0/2	0/2	0/2	0/2	N
4	*N. tabacum* 'Samsun' + CPMV-GFP + PVY	Suppression of silencing by potyviral HC-Pro	0/2	0/2	0/2	0/2	0/2	0/2
5	*N. tabacum* 'Samsun' + CPMV-GFP + TSWV	Suppression of silencing by tospoviral NSs	0/2	0/2	N	N	N	N
6	*N. tabacum* 'Samsun' ATTA CMV 2b + CPMV-GFP	Suppression of silencing by cucumoviral 2b	0/2	0/2	0/2	0/2	0/2	N
7	*N. tabacum* 'Samsun' + CPMV-GFP + CMV	Suppression of silencing by cucumoviral 2b	0/2	0/2	0/2	0/2	0/2	0/3
8	SH-L(4) *N. tabacum* 'Samsun NN' + CPMV-GFP	No accumulation of salicyilic acid	0/2	0/2	0/2	0/2	0/2	0/2
9	NahG-10 *N. tabacum* 'Xanthi nc' + CPMV-GFP	No accumulation of salicyilic acid	0/2	0/2	0/2	0/2	0/2	0/2
10	Tetr-18 *N. tabacum* 'Samsun NN' + CPMV-GFP	Ethylene-insensitivity	0/2	0/2	0/2	0/2	0/2	0/2

(a) The incubation temperature was 23 °C, unless stated otherwise. One lower leaf/plant was inoculated with purified CPMV-GFP virions or with leaf extracts of PVY, TSWV or CMV infected plants. The inoculated lower leaves from all plants were infected until 21 dpi. ATTA, agroinfiltration.

(b) Systemic infection of non-inoculated upper leaves was checked by GFP fluorescence and back inoculation of extracts of each upper leaf onto a cowpea plant. PVY, TSWV and CMV infection was checked based on symptoms in *N. tabacum*. Number of upper leaves analysed per time point as indicated in Table 1.

N Not determined because plants or inoculated leaves did not survive.

Neither incubation temperature, RNA silencing-, salicylic acid- nor ethylene-mediated resistance are the limiting factors for CPMV systemic infection of *N. tabacum*

To test the hypothesis that incubation temperature would somehow influence CPMV systemic spread in *N. tabacum* plants, CPMV-GFP inoculated *N. tabacum* were incubated at 32 °C or 15 °C, instead of 23 °C as performed for all other experiments described. In a time-course, non-inoculated upper leaves were scrutinized for CPMV-GFP infection by recording GFP fluorescence and by back inoculation onto cowpea plants. At 10 dpi the inoculated leaves of plants incubated at 15 °C presented necrotic lesions (Fig. 2a and 2b) and CPMV-GFP fluorescent foci (Fig. 2c). The necrotic lesions were associated with the CPMV-GFP infected areas, while non-infected areas of the inoculated leaves were totally free of necrosis (Fig. 2b and 2c). Necrotic lesions were never observed in mock-inoculated plants incubated at 15 °C or in CPMV-GFP-inoculated plants incubated at 32 °C (data not shown). Incubation of plants at temperatures higher or lower than 23 °C did not result in CPMV systemic spread (Table 4, Treatment 1 and 2).

Considering the premise that RNA silencing somehow may oppose CPMV infection in *N. tabacum*, we tested whether suppressors of RNA silencing from heterologous viruses, i.e. potyviral HC-Pro, tospoviral NSs, or cucumoviral 2b, would promote systemic spread of CPMV-GFP in *N. tabacum* plants. HC-Pro was delivered either by agroinfiltration with a CABMV HC-Pro construct (Mlotshwa *et al.*, 2002b) or by co-infection with PVY. NSs was delivered by inoculation with TSWV, while CMV 2b was delivered either by agroinfiltration of a 2b encoding construct (Bucher *et al.*, 2003) or by inoculation with CMV. Agroinfiltration of a GFP-binary construct performed onto the abaxial surface of *N. tabacum* leaves efficiently resulted in expression of GFP on the adaxial epidermis cells (data not shown). This demonstrated that agroinfiltration is a suitable approach to deliver the suppressors on the adaxial epidermis cells of *N. tabacum*, where the mechanical inoculations with CPMV-GFP were performed. The effect of the various treatments on systemic infection of *N. tabacum* plants by CPMV-GFP was investigated in a time course up to 21 dpi. None of the three viral suppressors of RNA silencing (NSs, HC-Pro and 2b) promoted systemic spread of CPMV-GFP in *N. tabacum* plants (Table 4, Treatments 3-7), indicating that RNA silencing is not the crucial factor blocking CPMV systemic infection of *N. tabacum*.

Salicylic acid (SA) and ethylene have been reported to be involved in numerous signalling pathways leading to resistance of plants against invading microbes (reviewed by Dong, 1998), in some cases blocking the systemic infection process of pathogens (reviewed by Pieterse & van Loon, 1999; Heil & Bostock, 2002). To

investigate whether SA or ethylene play a role in the inability of CPMV to infect *N. tabacum* systemically, the spread of CPMV-GFP was examined in transgenic *N. tabacum* lines which do not accumulate SA or are ethylene-insensitive. Three lines were used: NahG-10 (Gaffney *et al.*, 1993) containing the bacterial *nahG* gene encoding salicylate hydroxylase which converts SA to catechol, SH-L(4) (Bi *et al.*, 1995) which encodes another bacterial salicylate hydroxylase homologous to *nahG*, and Tetr-18 (Knoester *et al.*, 1998) which encodes the gene *etr1-1* (mutant of the gene *etr1*) conferring insensitivity to ethylene. The line SH-L(4) presents a higher salicylate hydroxylase activity than NahG-10 (Mur *et al.*, 1997). All lines could be locally infected with CPMV-GFP, however none of the lines supported systemic spread (Table 4, Treatments 8-10).

Collectively, the results obtained indicate that incubation temperature, defence mechanisms based on RNA silencing, salicylic acid and ethylene, apparently do not influence the barrier against systemic infection of *N. tabacum* plants by CPMV.

Table 5. CPMV infection of *N. benthamiana* scion grafted on *N. tabacum* cv. Samsun rootstock.

Inoculation of rootstock	Local infection of inoculated rootstock[(a)]	Systemic infection of scion[(b)]
CPMV-GFP	9/9	0/9
CPMV-WT	9/9	0/9
CMV	4/4	4/4

(a) Nr. of infected rootstocks (9 dpi) per total Nr. of grafts performed; based on symptoms for CMV, on GFP fluorescence for CPMV-GFP and on back inoculation of rootstock leaf extracts on cowpea plants for CPMV-WT.
(b) Nr. of systemically infected scions (9 dpi) per total Nr. of grafts performed; based on back inoculation of scion extracts on cowpea plants.

CPMV is incapable of phloem loading in *N. tabacum*

The experimental evidence presented so far indicates extensive replication and cell-to-cell movement of CPMV in inoculated *N. tabacum* leaves, but no systemic infection and the latter is not likely to be due to host defence reactions. Rather, there appears to be a blockage in vascular spread possibly based on the absence of suitable host factors that support systemic movement. To determine whether CPMV could be loaded into the phloem of *N. tabacum*, *N. benthamiana* scions were grafted on CPMV-infected *N. tabacum* rootstocks. If CPMV would be loaded into the phloem of *N. tabacum* rootstocks, the virus would go along the phloem stream to the susceptible *N. benthamiana* scion, where it can be unloaded and establish infection. Neither CPMV-WT nor CPMV-GFP were ever detected in *N. benthamiana* scions grafted on virus-

inoculated *N. tabacum* rootstocks (Table 5). Controls consisted of similar grafts whose rootstocks were inoculated with CMV, a virus that is able to infect *N. tabacum* systemically. As expected, CMV infection was found to take place in the scions (Table 5). These results indicate that CPMV is not loaded into the *N. tabacum* phloem.

DISCUSSION

Different plant species of the genus *Nicotiana* (tobacco) apparently support CPMV-infection to different extents. The virus can infect *Nicotiana benthamiana* systemically, whereas *Nicotiana tabacum* is only locally infected, i.e. in the inoculated leaf (this report; Huber *et al.*, 1977; Nida *et al.*, 1992). Although CPMV does not induce visible symptoms in *N. tabacum* plants, it replicates to high concentration in inoculated leaves (Nida *et al.*, 1992) and protoplasts (Huber *et al.*, 1977). CPMV infected *N. tabacum* cells produced typical cytopathic structures (Huber *et al.*, 1977) found also in infected cowpea cells (de Zoeten *et al.*, 1974; Carette *et al.*, 2002b). The replication of CPMV in *N. tabacum* protoplasts (Huber *et al.*, 1977) has a longer lag period (around 10 hours more) than in cowpea protoplasts (Hibi *et al.*, 1975). Although CPMV replicates slower in *N. tabacum* protoplasts, it eventually reaches virus titres comparable to those in cowpea protoplasts (Hibi *et al.*, 1975; Huber *et al.*, 1977). Here it was observed that *N. tabacum* requires high inoculum pressure for successful CPMV infection of inoculated leaves. Moreover, GFP fluorescence (indirect indication of CPMV-GFP replication and accumulation) was observed to decrease faster with time in *N. tabacum* than in *N. benthamiana* inoculated leaves. This all points to some opposition against CPMV replication and/or accumulation in *N. tabacum* due to unknown mechanisms. Nevertheless, the virus is capable of extensive local spread throughout several cell types and even attains vicinities of the vascular tissue in the infected leaves.

Several experiments were carried out to get a clue to the mechanism that blocks systemic spread of CPMV in *N. tabacum*. A time-course infection of upper leaves of plants that were previously either mock- or CPMV-inoculated on their lower leaves showed that CPMV was able to infect the upper leaves to the same extent in both cases. This suggests that there is no systemic resistance signal impeding CPMV systemic spread within *N. tabacum* plants. Mock inoculation of lower leaves was an essential control for this experiment, since local and systemic defence responses are known to be triggered after wounding (reviewed by Pieterse & van Loon, 1999). Also, the experiments with ethylene-insensitive transgenic tobacco (line Tert-18) and tobacco plants incapable of SA accumulation (lines SH-L(4) and NahG-10), aimed to detect SA- or ethylene-mediated host responses, were negative. The hindrance of CPMV systemic infection probably already takes place in the inoculated leaf, without

apparent disturbance of viral local spread. For several other plant viruses, interference of infection by SA- or ethylene-induced responses has been reported. Both are part of different signal transduction pathways which can lead to blockage of systemic virus infection (Verberne *et al.*, 2003; reviewed by Murphy *et al.*, 2001). For example, SA signalling results in blockage of *Tobacco mosaic virus* (TMV) systemic spread in tobacco plants expressing the *N* resistance gene by a mechanism denoted systemic acquired resistance (SAR) (Ross, 1961; Malamy *et al.*, 1990). SA also reduces TMV replication (Chivasa *et al.*, 1997, Naylor *et al.*, 1998) and the rate of TMV cell-to-cell movement through mesophyll cells (Murphy & Carr, 2002). In CMV case, SA does not affect viral replication (Naylor *et al.*, 1998) or cell-to-cell movement (Murphy & Carr, 2002), but it interferes with viral systemic spread, not involving SAR (Ji & Ding, 2001). SA probably also interferes with RNA silencing-related factors since a *N. tabacum* RNA-dependent RNA polymerase (RdRP), enzyme involved in initiation and amplification of RNA silencing, was found to be SA-inducible (Xie *et al.*, 2001).

Different incubation temperatures (15, 23 or 32 °C) did not affect systemic spread of CPMV in *N. tabacum* plants. However, at 15 °C CPMV-inoculated *N. tabacum* leaves developed necrotic lesions that were not observed in inoculated leaves kept at higher temperatures or mock-inoculated leaves kept at 15 °C. No clear explanation for this phenomenon can be given yet. The effect of high and low temperatures on virus infection varies largely among various plant-virus systems. For instance, TSWV symptoms were more severe and systemic spread faster at high than at low temperature regime in *N. tabacum* and *D. stramonium*, but in *P. ixocarpa* the speed of systemic spread remained unaltered (Llamas-Llamas *et al.*, 1998). At low temperatures (15 or 21 °C) *N. benthamiana* plants were more susceptible to *Cymbidium ringspot virus* (CymRSV). This was attributed to inhibition of RNA silencing (Szittya *et al.*, 2003) as the levels of viral siRNAs (the key molecules of RNA silencing mediated-defence pathways) decreased in these plants. Consistently, RNA silencing was active and the amount of CymRSV siRNAs increased with rising temperatures (24 or 27 °C). In the CPMV-*N. tabacum* system it is also possible that low temperature favoured the virus local infection by suppressing a resistance mechanism (e.g. RNA silencing) existing in the plant, but this was still not sufficient to promote the virus systemic spread.

To investigate a role of RNA silencing in hampering systemic spread of CPMV in *N. tabacum*, the effect of different heterologous suppressors of silencing on CPMV systemic infection was tested. HC-Pro and NSs suppress a maintenance step of RNA silencing resulting in prolongation of viral RNA replication (Kasschau *et al*., 1997; Pruss *et al*., 1997), and 2b suppresses a systemic signalling step in the silencing pathway (Brigneti *et al*., 1998). No obvious improvement of infection, such as earlier or stronger CPMV-GFP fluorescence, was observed in the presence of the suppressors

and the suppressors did not evoke systemic spread of CPMV. Like in *N. benthamiana* (Chapter 4 of this thesis), also in *N. tabacum* HC-Pro and NSs significantly increased the number of infection foci upon RNA inoculation (data not shown). This demonstrates that RNA silencing plays - at least quantitatively - a role in the establishment of CPMV infection in the semi-permissive host *N. tabacum*, as it does in the permissive host *N. benthamiana*.

In grafting experiments, *N. benthamiana* scions did not become infected by CPMV-infected *N. tabacum* rootstocks. As *N. benthamiana* is a systemic host for CPMV and the virus can be unloaded from its phloem, this indicates that the virus is not loaded into the phloem of *N. tabacum*. Plant resistance to systemic infection as a result of impaired phloem loading or unloading has been described for several virus-plant systems. Goodrick *et al.* (1991) showed that *Cowpea chlorotic mottle bromovirus* (CCMV) was able to move from cell-to-cell in the inoculated leaf in soybean genotype PI 346304, but could not pass from bundle sheath cells to other phloem-associated cells. *Red clover necrotic mosaic dianthovirus* (RCNMV) systemically infects *N. benthamiana*, *N. edwardsonii* and cowpea, but is unable to be loaded into the phloem of *N. tabacum* (Wang *et al.*, 1998). It was found that in the latter plant, RCNMV can infect bundle sheath and phloem parenchyma cells, but not the companion cell-sieve element (CC-SE) complex. Schaad & Carrington (1996) demonstrated that *Tobacco etch potyvirus* (TEV) was restricted in its ability to move systemically in *N. tabacum* line V20 even though virus was detected in phloem parenchyma and CCs, suggesting blockage of TEV loading into or unloading from the SEs. The genetic basis for the V20 restriction is due to the interaction of recessive genes at two non-linked loci. There are two other examples of recessive traits, which do not disturb local spread or cause hypersensitive response (HR) controlled cell death in inoculated leaves, despite blocking virus phloem loading or unloading by unknown mechanisms: the mutation *vsm1*, which restricts *Turnip vein clearing virus* (TVCV) systemic spread in *Arabidopsis thaliana* (Lartey *et al.*, 1998), and the *va* gene, that blocks vascular movement of *Potato virus A* (PVA) in potatoes (Hämäläinen *et al.*, 2000).

In a few cases, the genetic basis of resistance to virus vascular movement is better understood. For instance, *A. thaliana* ecotype Columbia specifically restricts the vascular movement of TEV without involving HR or SAR, and is associated with at least two dominant genes denoted *RTM1* and *RTM2* (Mahajan *et al.*, 1998; Whitham *et al.*, 1999). RTM1/RTM2-restriction was shown to be SA-independent (Mahajan *et al.*, 1998) and specific to TEV. *RTM1* encodes a lectin-like protein with similarity to several proteins that have diverse roles in insect and pathogen defence (Chisholm *et al.*, 2000). *RTM2* encodes a protein with a domain similar to plant small heat shock proteins (HSPs), though RTM2 does not possess typical chaperone functions, i.e. it is

neither heat inducible nor involved in thermotolerance (Whitham *et al.*, 2000). RTM1 protein accumulates in SEs and in immediately adjacent cells (probably CCs), whereas RTM2 is detected exclusively in SEs (Chisholm *et al*., 2001). Different hypotheses were proposed (Whitham *et al.*, 2000; Chisholm *et al*., 2001) for how the RTM system could function within the SE to restrict TEV vascular movement. RTM1 and/or RTM2 could interact with and inactivate viral proteins (i.e. HC-Pro, CI, NIa and CPs) or host proteins necessary for TEV vascular movement. Alternatively, RTM1 and/or RTM2 might be necessary to produce, transport or perceive a signal that results in establishment of a TEV-restrictive state in the systemic tissue. In *N. tabacum*, the cadmium-induced glycine-rich protein (cdiGRP) blocks the systemic spread of TVCV in a SA-independent manner (Citovsky *et al.*, 1998; Ueki & Citovsky, 2002). cdiGRP is expressed in the cell wall exclusively at the vascular tissue and exerts its inhibitory effect on TVCV vascular movement by enhancing callose deposits in the phloem, thus blocking the virus phloem unloading (Ueki & Citovsky, 2002). The callose deposits probably negatively regulates the ability of plasmodesmata to allow passage of virus.

In the *N. tabacum*-CPMV system, the involvement of specific host factors in vascular movement still remains to be determined. It could be that host factors actively block vascular movement or, that the absence of host factors essential for virus loading disables the vascular movement. Since the grafts indicate that CPMV vascular movement in *N. tabacum* is blocked at the phloem loading side it is important to determine at which cell type within the phloem boundary CPMV infection stops, i.e. whether it is able to reach the CC-SE complex or not. Maybe *N. tabacum* lacks a protein function essential for the interaction of CPMV within plasmodesmata at the boundary between the phloem and the non-vascular tissue (i.e. bundle sheath-phloem parenchyma) or within the pore-plasmodesma units in the phloem (i.e. PPUs, the special plasmodesmata connecting CCs to SEs). As suggested for the Arabidopsis-TEV RTM system, *N. tabacum* may express factors that sequester viral and/or host proteins necessary for CPMV vascular movement. Alternatively, as in the *N. tabacum*-TVCV cdiGRP system, *N. tabacum* might trigger an active response that specifically blocks CPMV phloem loading. However *N. tabacum* does not seem to produce systemic signalling factors that impede the CPMV systemic spread. These findings make *N. tabacum* an interesting system for investigations of the host factors involved in preventing CPMV vascular movement, as compared to host factors that promote viral vascular movement in CPMV permissive hosts.

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CHAPTER 6

General Discussion

In this thesis the mechanisms underlying the systemic infection of plants by *Cowpea mosaic virus* (CPMV) were investigated. To accomplish systemic infection plant viruses must replicate, move locally (cell-to-cell movement) and systemically via the vasculature (vascular movement) within a plant host. For CPMV, the intracellular replication cycle as well as cell-to-cell movement are processes that are reasonably well understood (PhD thesis by van Bokhoven, 1993; Kasteel, 1999; Bertens, 2000; Carette, 2002; Carvalho, 2003; reviewed by Pouwels *et al*., 2002a). In view of the knowledge gap on the mechanism of CPMV vascular movement, as well as host barriers and defence responses encountered by the virus during this process, the research reported in this thesis concentrated on these aspects of the viral infection process.

Generation of virus mutants by reverse genetics is currently the most common approach to study the viral factors necessary for vascular movement. For most viruses, deletion of or mutations in the viral coat protein (CP) are associated with disturbance or even absence of vascular movement. Some viruses that require the CP for systemic spread move as virions and others as nucleoprotein complexed with the CP in non-virion form through the phloem, while viruses that do not require the CP move through the vasculature necessarily as a non-virion nucleoprotein complex (reviewed in Chapter 1). Since CPMV requires its movement protein (MP) and both its small and large CPs for tubule-guided cell-to-cell movement (Wellink & van Kammen, 1989; van Lent *et al.*, 1990), deletion of any of the genes encoding these proteins results in impeded local spread, the viral infection thus becoming restricted to a single cell (Verver *et al*., 1998). Consequently, studying the possible enrolment of one of these proteins in vascular movement of CPMV by reverse genetics presents major difficulties. To date, no CPMV MP or CP mutant or recombinant has been obtained, which is specifically disturbed in its vascular movement function without a defect in cell-to-cell movement (Bertens *et al.*, 2000; Gopinath *et al.*, 2000). In Chapter 3, in an attempt to resolve this technical difficulty, the CPs were provided *in trans* to complement the cell-to-cell movement of a CPMV mutant devoid of CPs. In this deletion mutant (CPMV-ΔCP), the genes encoding the CPs were replaced by the GFP reporter gene to facilitate *in vivo* observation of virus spread (Verver *et al*., 1998). Since no systemically infectable (i.e. permissive) host plant, transgenically expressing the CPs was available, these proteins were provided via *Agrobacterium* T-DNA

transient-expression assay (ATTA or agroinfiltration) performed on the inoculated leaves of the permissive experimental host *N. benthamiana*. The natural host for CPMV, cowpea, was not useful in view of the poor efficiency of agroinfiltration of this plant species. The intention was to complement the inability of CPMV-ΔCP cell-to-cell movement within the inoculated leaf and to observe whether the mutant would exit from the vascular tissues in the upper leaves in the absence of the CPs. While the *trans* complementation led to successful CPMV-ΔCP cell-to-cell movement *in planta*, it was apparently not sufficient to allow the mutant virus to reach and enter the vasculature, thus the virus exit from the vasculature of upper leaves could not be analysed. To resolve this technical challenge, design of a permissive host that transgenically expresses the CPs is essential. In view of the difficulties to transform and regenerate cowpea, *N. benthamiana* would be a more suitable plant species for transformation (Sijen *et al.*, 1995).

To get insight in the form in which CPMV (as virion or otherwise) circulates along the vasculature, vascular sap (containing both xylem and phloem contents) was collected from CPMV-infected cowpea plants and analysed in western blots probed with various antisera against CPMV proteins (Chapter 3). The only viral factors detected in the vascular sap were the CPs, indicating that the CPs are required for CPMV vascular movement, either as virions or as ribonucleoprotein complex in a non-virion form. To discriminate whether the CPs detected in vascular sap originated from the phloem and/or xylem different methods were used to collect phloem or xylem sap separately from CPMV-infected cowpeas, i.e. the $CaCl_2$-method to collect xylem sap (Schobert & Komor, 1990), the EDTA-method (King & Zeevaart, 1974; Lejeune *et al.*, 1988) and honeydew from feeding aphids (Wilkinson *et al.*, 1997) to collect phloem sap. The stylectomy method (Fujimaki *et al.*, 2000) to collect phloem sap was not applied because it is not suitable for legume species such as cowpea (Tjallingii, personal communication). Despite exhaustive attempts, the volume of the samples obtained was minimal or the viral proteins remained under the detection level in western blots. To assess whether the CPs detected in the vascular sap were in a virion form or not, grafting experiments were done using immune cowpea cv. TVu-470 plants (TVU) as scions grafted onto permissive CPMV-infected cowpea cv. California Blackeye (CBE). Due to TVU immunity (i.e. no viral replication *in planta*), any infectious entity (i.e. virion or RNA complexed with CPs) detected in these scions should originate from vascular transport from the infected CBE rootstock. CPMV virions were consistently found in extracts of TVU scion midveins, indicating that CPMV moves systemically as virions. Nevertheless, it was not possible to unequivocally discriminate whether these virions were located in the phloem or in the xylem. Virions could enter the xylem in the infected root system of the CBE rootstock

as a result of infection of undifferentiated xylem cells as suggested by French & Elder (1999). To resolve this it is imperative to repeat the graft experiments using TVU as rootstock, CPMV-infected CBE as intergraft and TVU as scion. In this way, the root system of the grafts (i.e. immune TVU rootstock) cannot be a source of xylem-residing virions. Virions eventually found in the TVU scion would then originate from the phloem of the CPMV-infected CBE intergraft.

The occurrence of viruses in xylem does not necessarily result in the spread of infection to healthy parts of the plant (Caldwell, 1931; French *et al.*, 1993; French & Elder, 1999), and the phloem is the significant route for systemic spread of the great majority of plant viruses. Obvious exceptions are viruses that are transmitted by soil-fungi, which inoculate virus directly in the roots and not in the aerial parts of the plant (Tamada, 1975; Putz, 1977; Tamada & Kuzume, 1991). Also CPMV is thought to be delivered into the xylem by its beetle vector (reviewed by Gergerich, 2001). Nevertheless, upon gross-wound inoculation (Gergerich *et al.*, 1983) of cowpea, which mimics beetle transmission, a GFP-expressing CPMV (CPMV-GFP) spread systemically in the same pattern as photo-assimilates do (Chapter 3), i.e. non-inoculated leaves that had undergone sink-source transition did not get infected. Considering these experiments, it is plausible that even upon beetle transmission CPMV rapidly reaches the phloem and that also in this case the phloem is the effective route for vascular movement. In Chapter 2 (Silva *et al.*, 2002) it was further shown that upon mechanical inoculation onto the adaxial epidermis of cowpea leaves, CPMV moves from cell-to-cell to the vasculature where it enters (loading) the phloem of minor veins. Subsequently, the virus exits (unloading) exclusively from major veins. As typical for phloem transport of viruses (Leisner *et al.*, 1992, 1993; Cheng *et al*., 2000; Oparka & Santa-Cruz, 2000; Roberts *et al.*, 1997), also CPMV does not infect leaves that had undergone complete transition from sink to source and it infects strong sink tissues first, i.e. roots.

Detailed analysis of serial sections from loading sites in minor veins and unloading sites in major veins of cowpea showed a remarkable absence of CPMV replication in the companion cell (CC), while replication clearly occurred in the phloem parenchyma (PPC) and bundle sheath cells (BSC). The absence of virus infection in CCs in inoculated leaves was also reported for *Sunn-hemp mosaic tobamovirus* (SHMV) in *Phaseolus vulgaris* and *Pisum sativum* (Ding *et al*., 1998). Nevertheless, in the majority of the cases studied, viruses were detected in the CC of both loading and unloading sites. For instance, *Potato Y potyvirus* and *Peanut stripe potyvirus* in *N. benthamiana*, as well as for *Tobacco mosaic tobamovirus* in *N. benthamiana*, *Capsicum annuum* and *Lycopersicon esculentum* were observed in CCs within the inoculated leaves (Ding *et al*., 1998). *Potato X potexvirus* in *N.*

benthamiana (Roberts *et al*., 1997), *Bean dwarf mosaic begomovirus* in *P. vulgaris* (Wang, H.-L. *et al.*, 1996) and SHMV in *P. sativum* (Ding *et al.*, 1998) were equally detected in CCs within the upper non inoculated leaves.

It was suggested (reviewed by Santa-Cruz, 1999) that some viruses exploit the plasmodesmata between sieve element (SE) and PPC to gain access to the phloem, rather than entering the CC directly. Considering that no plasmodesmata were ever found between PPC and SE in cowpea veins, this route for phloem loading is less likely for CPMV in this particular host. The symplasmic connection between SE via CC with surrounding vascular cells suggests a role of the CC in loading and unloading of photosynthate and also CPMV in cowpea. The fact that no virus was detected in CC could indicate that CPMV is loaded from CC into SE in a non-virion form or that virions were under detectable level. No CPMV virions or viral antigens were detected *in situ* in the SE by electron microscopy.

To assess whether CPMV would employ the tubule-guided mechanism (typical of cell-to-cell movement within mesophyll cells) also within the phloem tissue, in Chapter 2 the presence of tubular structures transporting CPMV virions was surveyed in the plasmodesmata connecting different phloem cell types. Tubular structures transporting CPMV virions were found in the phloem exclusively in BSC-BSC, BSC-PPC and PPC-PPC boundaries but were never observed within the core of the phloem, i.e. beyond the PPC boundary, between PPC-CC or CC-SE. It has been demonstrated for several plant species that the plasmodesma connecting the CC-SE complex (denoted pore-plasmodesma unit - PPU) differ from those in mesophyll cells and may allow the passage of large molecules (Kempers & van Bel, 1997; van Bel, 1996; Turgeon, 2000; Fisher & Cash-Clark, 2000), and even whole virions (Esau & Hoefert, 1972; Murant & Roberts, 1979; D'Arcy & Zoeten, 1979; Shepardson *et al.*, 1980; Mutterer *et al.*, 1999). Establishing the size exclusion limit (SEL) of PPU in cowpea source (that load the virus into phloem) and sink (that unload the virus from phloem) tissues would indicate whether virions could or could not be transported through these specialised plasmodesmata without gating (modifying the SEL) them. In contrary to the precedents for other viruses in the literature (Esau & Hoefert, 1972; Murant & Roberts, 1979; D'Arcy & Zoeten, 1979; Shepardson *et al.*, 1980; Mutterer *et al.*, 1999), CPMV virions were never observed in the PPUs of cowpea leaves. It is possible that CPMV virions are loaded into and unloaded from cowpea veins without gating the PPU, but this event may be transient and therefore escaped detection in the electron microscopy preparations.

The viral factors involved in CPMV vascular movement are barely known (Chapter 2 and 3), whereas the host determinants and factors affecting the systemic spread of this virus are completely obscure. In Chapter 5, it was tested whether semi-

permissive hosts, i.e. which do not allow all the steps necessary for systemic infection to take place, could be a useful tool to further unravel the mechanism of systemic spread by CPMV. *N. tabacum* is such a semi-permissive host, which supports CPMV replication and local cell-to-cell movement, but not the systemic infection (Huber *et al.*, 1977; Nida *et al.*, 1992). The most remarkable finding was that this plant, without noticeable disturbance in cell-to-cell movement, does not support CPMV phloem loading. No evidence was found for the presence of any systemic resistance signal, i.e. those involving either the hormones salicylic acid/ethylene (reviewed by Dong, 1998) or RNA silencing (reviewed by Yu & Kumar, 2003), that would result in the impediment of CPMV systemic infection of *N. tabacum* upper leaves. Several scenarios then may explain the lack of phloem loading. Firstly, there might be a quantitative effect in that, compared to a fully permissive host, the amount of viruses ultimately reaching the vasculature may be too low for successful loading. Secondly, phloem loading is a specific process requiring support by specific host components and some of these host components may not be compatible with CPMV infection in *N. tabacum*. To better understand the mechanisms of CPMV hampered phloem loading, it will be essential to determine what is the ultimate cell boundary (i.e. mesophyll cells-BSC; BSC-PPC, PPC-CC or CC-SE) that CPMV reaches within the phloem of inoculated *N. tabacum* leaves. If CPMV reaches the CC, it could be that *N. tabacum* lacks at least one essential factor that mediates CPMV phloem loading in permissive hosts, e.g. a protein that docks the virus to or mediates the virus transport through the PPU. Alternatively, it can be that in *N. tabacum* there are host factors involved in a resistance mechanism specifically active within the phloem that hinders the virus vascular loading, as it has been suggested for other non systemic host-virus combinations (Citovsky *et al.*, 1998; Chisholm *et al.*, 2000; Ueki & Citovsky, 2002). These findings make *N. tabacum* an interesting system for investigations of the host factors involved in preventing CPMV vascular movement, as compared to host factors that promote viral vascular movement in CPMV permissive hosts. Such investigations could be done, for instance, by genomics approaches using microarray technology (microchips) to compare the difference in ratios of mRNA expression of homologous genes between CPMV infected-*N. tabacum* (versus mock infected control) and CPMV infected-permissive host for this virus, such as *N. benthamiana* or *Medicago trunculata*, (versus mock infected control). Differentially expressed genes of this indirect comparison could be candidate host factors involved in CPMV vascular movement. To refine the information from genetics using cDNA microchips representing the whole plant genome, the microchips could be targeted with mRNAs from vascular tissue enriched samples. The interpretation of the outcome of such investigations would be aided by the rapidly increasing knowledge about the

plasmodesma and phloem specific factors in various plant species (Annals of the International Conference on Phloem Transport, Bayreuth-Germany, 2003).

Even in permissive hosts, CPMV is confronted with active defence responses against the viral infection process such as RNA silencing. In Chapter 4, the impact of RNA silencing on initiation of infection and local spread was studied *in planta* in the permissive host *N. benthamiana* by using heterologous suppressors of RNA silencing, i.e. the potyviral-HC-Pro (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998), tospoviral-NSs (Bucher *et al.*, 2003) and cucumoviral-2b (Brigneti *et al*., 1998). Upon inoculation with CPMV *in vitro* transcripts, HC-Pro and NSs, but not 2b, significantly increased the number of CPMV primary infection foci. These results indicate that RNA silencing already has an impact on infection at a very early stage. The different effects observed with suppressors HC-Pro and NSs on one hand and 2b on the other is probably due to fact that 2b suppresses a systemic signalling step in the silencing pathway (Brigneti *et al.,* 1998), while HC-Pro and NSs operate more promptly by suppressing a maintenance step of RNA silencing, which results in prolongation of viral RNA replication (Kasschau *et al.,* 1997; Pruss *et al*., 1997). Strikingly, the stimulating effect of the viral suppressors was not observed when virions were used as inoculum. This could be partially due to protection of the viral RNA by the CPs against non-specific degradation by nucleases, but could also be the result of suppression of RNA silencing by the CPs. Indeed, recently it was found that the small CP of CPMV is a suppressor of RNA silencing (Liu *et al.*, in press). Thus, in CPMV the encapsidation and suppression of RNA silencing functions are combined in the small CP, thereby equipping the virus with a biological advantage of being transmitted to plants in the immediate presence of its suppressor. Also in the case of potyviral transmission by aphids, HC-Pro is delivered to the plant cell together with the virions, as HC-Pro is a helper factor involved in aphid transmission (Thornbury *et al*., 1985) as well as an effective suppressor of silencing. The same holds for TSWV, a virus transmitted by thrips. Large amounts of the suppressor protein NSs are found in the salivary glands of thrips together with virions (Wijkamp *et al*., 1993), thus virions and NSs are probably injected simultaneously into the plant cell. Moreover, several virally encoded suppressors of RNA silencing have been shown to be essential factors in virus vascular movement (reviewed in Chapter 1). HC-Pro, for instance, facilitates transport of potyviral ribonucleoprotein complexed with CP in a non-virion form through plasmodesma into and out of SEs (Cronin *et al*., 1995; Roudet-Tavert *et al*., 2002). It may be that interaction of HC-Pro with potyviral RNA suppresses RNA silencing. Possibly as a result of the interaction with HC-Pro, the viral RNA escapes from the host defence mechanism and can be loaded into the phloem through the PPU, still interacting or not with HC-Pro (Kasschau & Carrington,

2001). With CPMV the CP(s) could function in a similar way to promote virus vascular movement. In contrast to their effect on the initiation of CPMV infection, the heterologous suppressors did not affect the rate of CPMV local spread, indicating that RNA silencing seems not to play a major role in this stage of the infection.

CPMV systemic infection appears to be a complex process. The results obtained in this thesis research represents a further refinement on the understanding of the mechanisms of CPMV systemic infection in its cowpea host, in particular with respect to the vascular movement. Upon introduction into cowpea cells, the virus is confronted with the host RNA silencing defence system, which is neutralized effectively by the viral suppressor *in casu* the small CP (Chapter 4 and Liu *et al.*, in press). Once infection is established, the virus spreads from cell-to-cell by tubule-guided virion transport (extensively studied in recent theses of Bertens, 2000 and Carvalho, 2003). Cell-to-cell transport in virion form also guarantees the simultaneous delivery of the viral suppressor, which is apparently required for successful infection in each cell. Tubule-guided movement delivers the virus eventually to the vasculature of minor veins, i.e. the phloem parenchyma cells. As in cowpea no plasmodesmata are present between the phloem parenchyma and sieve elements, CPMV is probably loaded into these elements via the companion cells by a so far unknown mechanism (Chapter 3). As virions have been found to circulate in the vasculature, it is feasible that this is also the infectious form that is loaded into the sieve elements and unloaded in the target leaves. The size exclusion limit of the specialized plasmodesmata between companion cells and sieve elements (PPUs), which most likely is larger than that of plasmodesmata in mesophyll cells. Hence, a mechanism such as tubule-guided virion transport may not be required for virion transport into sieve elements. Also, like with cell-to-cell movement, vascular movement of virions would ensure the immediate presence of the suppressor required for infection of cells in the target organ. However, the form in which CPMV is transported by the phloem is not unequivocally identified.

The ability of CPMV to move locally from cell-to-cell but not systemically in *N. tabacum* shows that these modes of transport are mechanistically different and indicates that phloem tissue in some way can present a specific barrier to the virus. As shown in Chapter 5, CPMV is not loaded into the phloem of *N. tabacum*. These observations point to the requirement for compatible host factors (e.g. within the PPUs) participating in vascular transport. Hence, comparative analysis of permissive and semi-permissive host plants could give more specific insights in the mechanism of successful systemic infection by CPMV and by plant viruses in general.

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Summary

Systemic virus infection of plants involves intracellular replication, local spread within the inoculated leaf (cell-to-cell movement), and subsequently, long-distance spread to other plant parts via the vasculature (vascular movement). Cell-to-cell movement occurs through the plasmodesma (PD), which are regulated channels in the cell wall connecting adjacent cells. The PD is modified by plant viral movement proteins (MP) to allow passage of a viral RNA-MP complex as happens with *Tobacco mosaic virus* (TMV), or virions as happens with *Cowpea mosaic virus* (CPMV). With the latter virus, virions move through tubules built-up from the MP (tubule-guided cell-to-cell movement). For vascular movement, viruses must enter (loading), translocate through, and exit (unloading) from the phloem. Phloem (un)loading occurs through specialized PD, named the pore-plasmodesma-unit (PPU), connecting the companion cell (CC) and sieve element (SE). The PPU allows passage of much larger molecules than mesophyll PD do. Because of the peculiarities inherent to phloem tissue (e.g. PPU), mechanisms of cell-to-cell movement are usually distinct from those of vascular movement (reviewed in Chapter 1) for the same virus. For instance, TMV requires the viral coat protein (CP) for transport of virions through PPU, but the CP is dispensable for cell-to-cell movement. The success of plant virus infection is also the consequence of an antagonistic balance between viral infection and plant host defence mechanisms that specifically target viral replication (e.g. RNA silencing), or movement (e.g. systemic acquired resistance).

In this research thesis CPMV was used as a model for investigations on the mechanisms of systemic infection of plants. Since CPMV replication and cell-to-cell movement are well-investigated, the thesis research was concentrated on vascular movement of CPMV and on barriers imposed by different plant species against systemic infection by this virus.

To examine the characteristics of vascular movement in *Vigna unguiculata* (cowpea), GFP-expressing CPMV (CPMV-GFP) was mechanically inoculated to primary leaves and infection was followed over time (Chapter 2). CPMV-GFP was loaded into both major and minor veins of the primary leaves and unloaded exclusively from major veins, preferentially class III, in the secondary leaves similar to the route of photo-assimilates via phloem. Using electron microscopy, virus infection was observed in all vascular cell types of the loading and unloading sites, with the exception of CC and SE. Furthermore, tubules transporting virions were never found in the PD connecting phloem parenchyma cells (PPC) and CC, or CC and SE (i.e. PPU). Since in cowpea the SE is symplasmically connected only to the CC, these

observations suggest that, unlike cell-to-cell movement, CPMV vascular movement is not tubule-guided.

Mutational analysis by reverse genetics is the most common approach to the study of viral factors necessary for vascular movement. CPMV requires its MP and both coat proteins (CPs) for tubule-guided cell-to-cell movement, deletion of any of these genes results in impeded local spread and this restriction severely hampers application of reverse genetics on CPMV for this purpose. In Chapter 3, an attempt was made to circumvent this problem by providing the CPs *in trans* by agroinfiltration in *N. benthamiana* to complement cell-to-cell movement of a CPMV mutant devoid of CPs (CPMV-ΔCP). The aim was to observe whether the mutant would exit from vascular tissue in the absence of CPs in the upper leaves. While *trans* complementation of CPMV-ΔCP cell-to-cell movement was demonstrated *in planta*, the extent of spread was not sufficient to allow CPMV-ΔCP phloem loading, thus the phloem unloading of the mutant within the upper leaves could not be analysed.

Immunoblot analysis of vascular sap from infected cowpea plants showed the presence solely of viral CPs. Furthermore, virions were found in the vasculature of CPMV-immune cowpea scions grafted on CPMV-inoculated susceptible rootstocks (Chapter 3). These results indicate that CPMV circulates in the vasculature in form of mature virions. However, it could not be unequivocally determined whether virions were located in the phloem or in the xylem. As systemic spread by xylem has been reported for beetle transmissible viruses like CPMV, beetle transmission was mimicked by gross-wound inoculation (Chapter 3). However, in this case, as with mechanical inoculation using an abrasive, CPMV spread systemically via the phloem, i.e. directed to sink-leaves solely like the flow of photo-assimilates. This confirms that phloem is the prevailing route for CPMV vascular movement.

The potential role of RNA silencing during establishment of infection by CPMV was studied in Chapter 4. Using GFP-expressing CPMV constructs and *N. benthamiana* as host, the number of infection foci was recorded in the absence or presence of different viral suppressors of RNA silencing, i.e. potyviral HC-Pro, tospoviral NSs and cucumoviral 2b. Upon inoculation with CPMV *in vitro* transcripts, HC-Pro and NSs, but not 2b, significantly increased the number of CPMV primary infection foci. These results indicate that RNA silencing already has an impact on the establishment of infection even at an early stage. Interestingly, the stimulating effect of suppressors was not observed upon inoculation with virions. This effect may be explained by the recent finding (Liu *et al*., in press) that the small (S) CP acts as a suppressor of RNA silencing. To assess the effect of RNA silencing on viral local spread, GFP-expressing CPMV constructs impaired in local spread were tested in the

presence or absence of HC-Pro or NSs. Neither of these proteins affected the progress of infection, indicating that RNA silencing does not play a major role in this stage.

In Chapter 5, *N. tabacum*, a semi-permissive host of CPMV, was used to further unravel the viral systemic infection process. CPMV does not infect *N. tabacum* systemically despite extensive local spread in inoculated leaves. It is shown that neither incubation temperature nor RNA silencing-, salicylic acid- or ethylene-mediated resistance mechanisms are the limiting factors for CPMV systemic infection. Although CPMV-infected *N. tabacum* plants are normally asymptomatic, symptoms (i.e. necrotic lesions) in the inoculated leaves were observed at low temperature (15 °C), but not systemic movement. Grafting experiments indicate that CPMV is not capable of phloem loading in *N. tabacum*, a finding that makes this plant species an interesting system for investigations of the host factors involved in CPMV vascular movement.

Finally, in Chapter 6 possible mechanisms of vascular movement of CPMV are presented based on the results obtained in this thesis. Moreover, the various virus-host interactions, which contribute to the success or failure of systemic infection, are put into perspective.

Samenvatting

Voor het bewerkstellingen van een volledige infectie van een plant, moeten virussen niet alleen in staat zijn om zich te vermenigvuldigen in de plantencel, maar zich vervolgens ook verspreiden naar naburige cellen (cel-cel transport) en , via het vaatweefsel, naar andere delen van de plant (lange-afstand transport). Het cel-cel transport van virusdeeltjes of het virale genoom verloopt via plasmodesmata in de celwand. Dit zijn gereguleerde kanaaltjes die, m.b.v. viraal gecodeerde transporteiwitten, voor dit doel worden gemodificeerd. Het lange-afstand transport verloopt in de meeste gevallen via het floeem, met name via de zeefvaten. Hiervoor moet het virus in staat zijn om vanuit het geïnfecteerde mesophylweefsel, via de bundelschede en de begeleidende cellen, de zeefvaten te bereiken en, na transport naar andere delen van de plant, ook weer te verlaten voor verdere verspreiding. De eigenschappen (o.a. de doorlaatbaarheid) van plasmodesmata tussen cellen van het vaatweefsel zijn anders dan die van plasmodesmata tussen mesophylcellen. Met name de plasmodesmata tussen begeleidende cellen en zeefvaten, ook wel "pore-plasmodesma-unit" (PPU) genoemd, zijn uniek wat betreft hun structuur en grote doorlaatbaarheid. De mechanismen van viraal cel-cel transport zijn derhalve niet noodzakelijkerwijs dezelfde als die van lange-afstand transport. Zo is bijvoorbeeld het manteleiwit van tabaksmozaïekvirus (TMV) noodzakelijk voor het virale transport door het floeem, maar niet voor transport van cel naar cel. Dit geeft aan dat TMV waarschijnlijk als compleet virusdeeltje door het zeefvat wordt getransporteerd, maar van cel naar cel in een andere vorm, waarschijnlijk een complex van viraal RNA en transporteiwit. Het cowpeamozaïekvirus (CPMV) hanteert weer een ander mechanisme voor transport door de plant. Dit virus heeft zijn manteleiwitten wel nodig voor cel-cel transport en wordt als compleet virusdeeltje getransporteerd via buizen, opgebouwd uit het virale transporteiwit, en die de virusdeeltjes door plasmodesmata heen leiden.

Uitgebreid onderzoek heeft inmiddels geleid tot een goed inzicht in het cel-cel transport van CPMV en de daarbij betrokken virale componenten, maar over het mechanisme van lange-afstand transport van dit virus is nog zeer weinig bekend. Het in dit proefschrift beschreven onderzoek had dan ook tot doel om meer inzicht te verwerven in dit aspect van de CPMV infectie in planten.

Allereerst werd de systemische verspreiding van CPMV vanuit primaire bladeren van de natuurlijke waardplant cowpea (*Vigna unguiculata* c.v. California Blackeye) naar andere plantendelen gevolgd, door gebruik te maken van een virale mutant die het groen-fluorescerende eiwit GFP (green fluorescent protein) kon produceren (CPMV-GFP, Hoofdstuk 2). Hierbij werd vastgesteld dat CPMV-GFP in elk type nerf (klasse I t/m III "major veins"; klasse IV en V "minor veins") van het geïnoculeerde primaire

blad het zeefvat kon bereiken voor verder transport, maar dat het in de secundaire bladeren de zeefvaten voornamelijk verliet via de klasse III nerven. Verder werd d.m.v. elektronenmicroscopische analyse vastgesteld dat zowel in nerven waar het virus in de zeefvaten werd opgenomen, als in nerven waar het virus de zeefvaten weer verliet, de begeleidende cellen geen (cytopathologische) kenmerken van virusinfectie vertoonden, terwijl dat wel het geval was in alle andere celtypen van het vaatweefsel. Aangezien in cowpea, de zeefvaten symplastisch zijn verbonden met de begeleidende cellen via de gespecialiseerde PPU, geven deze resultaten aan dat het mechanisme van virustransport vanuit de begeleidende cellen naar de zeefvaten en omgekeerd, niet verloopt via het voor o.a. mesophylcellen waargenomen transport door virusgeïnduceerde buizen.

Omdat bij CPMV infectie de manteleiwitten noodzakelijk zijn voor de verspreiding van cel naar cel, kon voor het bepalen van een eventuele functie van deze manteleiwitten bij het transport via het floeem geen gebruik gemaakt worden van mutant-analyse. Zowel virusmutanten deficiënt in hun manteleiwitten als in hun transporteiwit zouden immers al geblokkeerd zijn in het voorafgaande cel-cel transport. Teneinde de rol van manteleiwitten bij het floeemtransport, met name het proces van uittreding uit het floeem, alsnog te kunnen bepalen werden complementatie-experimenten uitgevoerd (Hoofdstuk 3). Hierbij werd een CPMV mutant zonder manteleiwitten na inoculatie *in trans* gecomplementeerd met manteleiwitten die via agroinfiltratie in het blad tot expressie werden gebracht. De CPMV mutant verspreidde zich weliswaar in het blad, maar de verspreiding was te beperkt om het vaatweefsel te bereiken.

In sap afkomstig uit het vaatweefsel van CPMV-geïnfecteerde cowpea planten werden d.m.v. immuno-analyse uitsluitend de manteleiwitten van het virus gevonden. Tevens werden in extracten van de hoofdnerven van secundaire bladeren van cowpea TVU470, een veredelingslijn die immuun is voor infectie met het virus, virusdeeltjes gevonden, indien deze waren geënt op de gevoelige en geïnfecteerde 'California Blackeye'. Deze resultaten tonen aan dat virusdeeltjes via het vaatweefsel door de plant circuleren, maar sluiten niet uit dat deze virusdeeltjes aanwezig zijn in het xyleem.

In Hoofdstuk 4 is onderzocht of "RNA silencing", een natuurlijk antiviraal afweermechanisme van de plant, een rol speelt bij de initiatie van CPMV infectie. Hiertoe werden *in vitro* transcripten (RNA) van de CPMV-GFP mutant geïnoculeerd op bladeren van *N. benthamiana* in aan- en afwezigheid van verschillende virale onderdrukkers van dit afweermechanisme (het potyvirale HC-Pro, het tospovirale NSs en het cucumovirale 2b). In aanwezigheid van HC-Pro en NSs, maar niet van 2b, bleek het aantal infectiehaarden op het geïnoculeerde blad aanmerkelijk groter te zijn.

Klaarblijkelijk heeft het afweermechanisme reeds in een vroeg stadium een behoorlijk negatief effect op virusinfectie. Opmerkelijk was dat het effect van de gebruikte onderdrukkers niet werd waargenomen als de bladeren werden geïnoculeerd met complete virusdeeltjes (bemanteld RNA). Dit kan worden verklaard indien (een van) de manteleiwitten van CPMV een functie hebben als virale onderdrukker van het afweermechanisme. Recentelijk is op een andere manier bevestigd (Liu *et al*., in press) dat het kleine manteleiwit van CPMV inderdaad deze functie heeft.

Om verder inzicht te krijgen in proces van systemische virusverspreiding is gebruik gemaakt van *N. tabacum* planten. CPMV kan zich in geïnoculeerde bladeren van deze plantensoort weliswaar van cel tot cel verspreiden, maar is vervolgens niet in staat een volledig systemische infectie te bewerkstelligen. Middels diverse experimenten werd aangetoond dat voor de hand liggende natuurlijke afweermechanismen, gebaseerd op "RNA silencing", salicylzuur of ethyleen, niet verantwoordelijk zijn voor deze beperkte infectie (Hoofdstuk 5). De waarneming dat enten van de gevoelige *N. benthamiana*, geplaatst op geïnoculeerde *N. tabacum* onderstammen, ook niet werden geïnfecteerd met CPMV, toont aan dat het CPMV niet in de zeefvaten van *N. tabacum* bladeren kan worden geladen voor verder transport.

Tenslotte worden in Hoofdstuk 6 de experimentele resultaten van dit proefschrift in een bredere context geplaatst en bediscussieerd.

About the author

Marilia Santos Silva was born on January 15th, 1974, in Belém-PA, Brazil. After finishing the academic high school she studied Agronomy at the Federal University of Viçosa (UFV, Brazil) and graduated in February 1997. During her stay at UFV (1992-1997), she was awarded two scholarships (one in Plant Molecular Biology and another in Plant Virology) from the Scientific Initiation program of the National Council for Scientific and Technological Development (CNPq, Brazil), under supervision of Dr Elizabeth Fontes from the Department of Biochemistry and Molecular Biology. Subsequently, she followed an MSc program (1997-1999) in the Department of Cell Biology at Brasília University (UnB, Brazil), under the supervision of Dr Renato Resende, with a scholarship awarded by the "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES, Brazil). She obtained the MSc degree in Molecular Biology in February 1999. Her MSc thesis dealt with studies of the diversity and structure of the tospoviral movement protein. From March 1999 until February 2000 she worked as a guest researcher in the Laboratory of Virology at Wageningen University (WUR, The Netherlands) in the research group of Prof. Dr Rob Goldbach, Dr Ir Jan van Lent and Dr Ir Joan Wellink, with grants from the Joint Dutch-Israeli Agricultural Research Program (DIARP). In the latter case, the investigations were performed on the cell-to-cell movement of *Cowpea mosaic virus*. In March 2000 she was awarded a 4-year scholarship by the Foundation for Earth and Life Sciences (ALW) of the Netherlands Organisation for Scientific Research (NWO) to carry out her PhD research at the Laboratory of Virology and the Laboratory of Molecular Biology at Wageningen University, under the supervision of Dr Ir J. van Lent, Dr Ir J. Wellink and Prof. Dr R. Goldbach, within the research project that resulted in this thesis.

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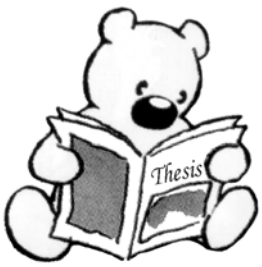
"The Lord loves his people and he crowns the humble with victory."
(Psalm 149, 4)

"*De fato o Senhor ama o seu povo e coroa de vitória os seus humildes."*
(Salmos 149, 4)

"De Heer schept behagen in zijn volk en de nederigen kroont Hij met zege."
(Psalmen 149,4)


Thesis

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