

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

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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 trifoliolate leaves. Phloem loading and unloading of CPMV was scrutinized 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 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.

Introduction

To successfully infect 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 utilization 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) utilizes 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).

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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.*, 1992; 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), that 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 RNA-2 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.

Methods

■ **Plants and protoplasts.** For plant studies, cowpea seeds (*Vigna unguiculata* cv. California Blackeye) were sown in sterilized soil and grown in a growth chamber with 16 h light at 23 °C. 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 with 16 h light at 23 °C. 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, M19GFP7 and M19GFP2A, expressing the green fluorescent protein (GFP) through different strategies (Fig. 1) were described previously (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

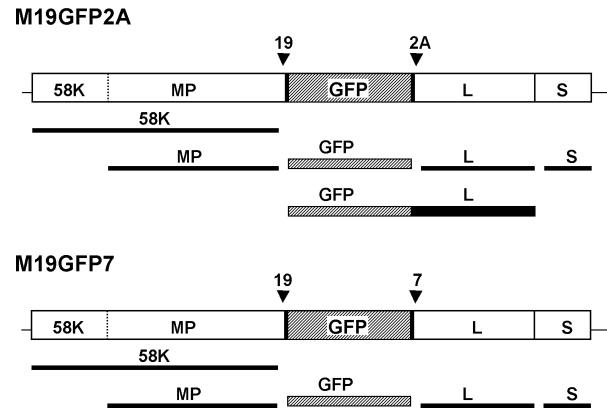


Fig. 1. Schematic representation of RNA-2 constructs of the recombinant viruses CPMV M19GFP2A and M19GFP7 and the corresponding expressed proteins. The green fluorescent protein (GFP) gene was inserted between the movement protein (MP) and the large coat protein (L) coding regions in an *in vitro* transcription 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 seven 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 L and 7 amino acids from the MP C terminus. M19GFP7 expresses free MP, GFP and L. S, small coat protein.

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 *Clai* to linearize the DNA, 1.25 mM of each rNTP (Promega, 25 mM each), 25 units of T7 RNA polymerase and its buffer at appropriate final concentration as suggested by the manufacturer. The reactions were incubated at 37 °C for 1–1.5 h. 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 coinoculated 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 1×10^6 protoplasts were inoculated with 5 µg each of RNA-1 and RNA-2 *in vitro* transcripts using polyethylene glycol (PEG mol. mass 6000) as described by Van Bokhoven *et al.* (1993). The protoplasts were then incubated under continuous illumination at 25 °C for 48 h 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 PBS was added to the pellets and the protoplasts were disrupted by repeated resuspension through a syringe with a large 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 (p.i.). Experiments were performed twice each for lamina flaps and midveins.

■ **Imaging fluorescence.** The spread of CPMV infection was monitored by imaging GFP fluorescence resulting from 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 visualized 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, 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 to assess 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 three 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 occurring at all). 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 12 h later 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.

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 days p.i. (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 at 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 having 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 (three plants observed per day)

Table 1. Systemic accumulation of CPMV in cowpea plants over time

–, Not infected based on GFP fluorescence detection; +, infected based on GFP fluorescence detection. ND, Not determined (either not yet developed or too small to be observed).

Plant part*	Days p.i.													
	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 trifoliolate leaf petiole	–	–	–	–	–	+	+	+	+	+	+	+	+	+
(g) 1st trifoliolate leaf petiolule	–	–	–	–	–	–	–	–	–	–	+	+	+	+
(h) 1st trifoliolate leaf	–	–	–	–	+	+	+	+	+	+	+	+	+	+
(i) Stem below 2nd trifoliolate leaf	ND	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–	–	–
(j) 2nd trifoliolate leaf petiole	ND	ND	ND	ND	–	–	–	–	–	–	–	–	+	+
(k) 2nd trifoliolate leaf petiolule	ND	ND	ND	ND	–	–	–	–	–	–	–	–	–	–
(l) 2nd trifoliolate leaf	ND	ND	ND	ND	–	–	–	–	–	+	+	+	+	+

* Parts of cowpea plant are schematically represented in Fig. 2.

(Table 1; Fig. 2). Infection was first observed in the inoculated leaf at 2 days p.i. 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 days p.i., and infection developed extensively in those tissues over 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 days p.i.). Only after 5 days p.i. 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 trifoliolate leaf, but virus was unloaded in the first trifoliolate leaf at 5 days p.i. (Table 1; Fig. 2h). The petiole of the first trifoliolate leaf showed fluorescence only after 6 days p.i. (Table 1; Fig. 2f and 2f'), whereas infection of tissues in the stem above the primary leaf and in the petiolule of the first trifoliolate leaf were first observed, respectively, 10 days p.i. (Fig. 2e) and 11 days p.i. (Fig. 2g) and onwards (Table 1; Fig. 2e' and 2g'). Similar to the first trifoliolate leaf, the second trifoliolate leaf was infected prior (10 days p.i. onwards; Table 1; Fig. 2l and 2l') to its petiole (13 days p.i. onwards, Table 1; Fig. 2j and 2j'). Unloading and infection within the stem between the first and second trifoliolate leaves and the petiolule of the second trifoliolate 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 days p.i. At 14 days p.i. plants were screened for systemic infection. Systemic infection of the trifoliolate leaves was established when the inoculated leaves were removed at 2 days p.i. or later, but not when the inoculated leaves were removed at 1 day p.i. (data not shown). These results demonstrate that by 2 days p.i. CPMV had been loaded into the primary leaf phloem and was transported into the stem.

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 organization of the veinal structure of cowpea leaves was visualized by labelling the xylem with Texas Red dextran (Fig. 3a). The veinal network of cowpea plants is organized 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 as 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).

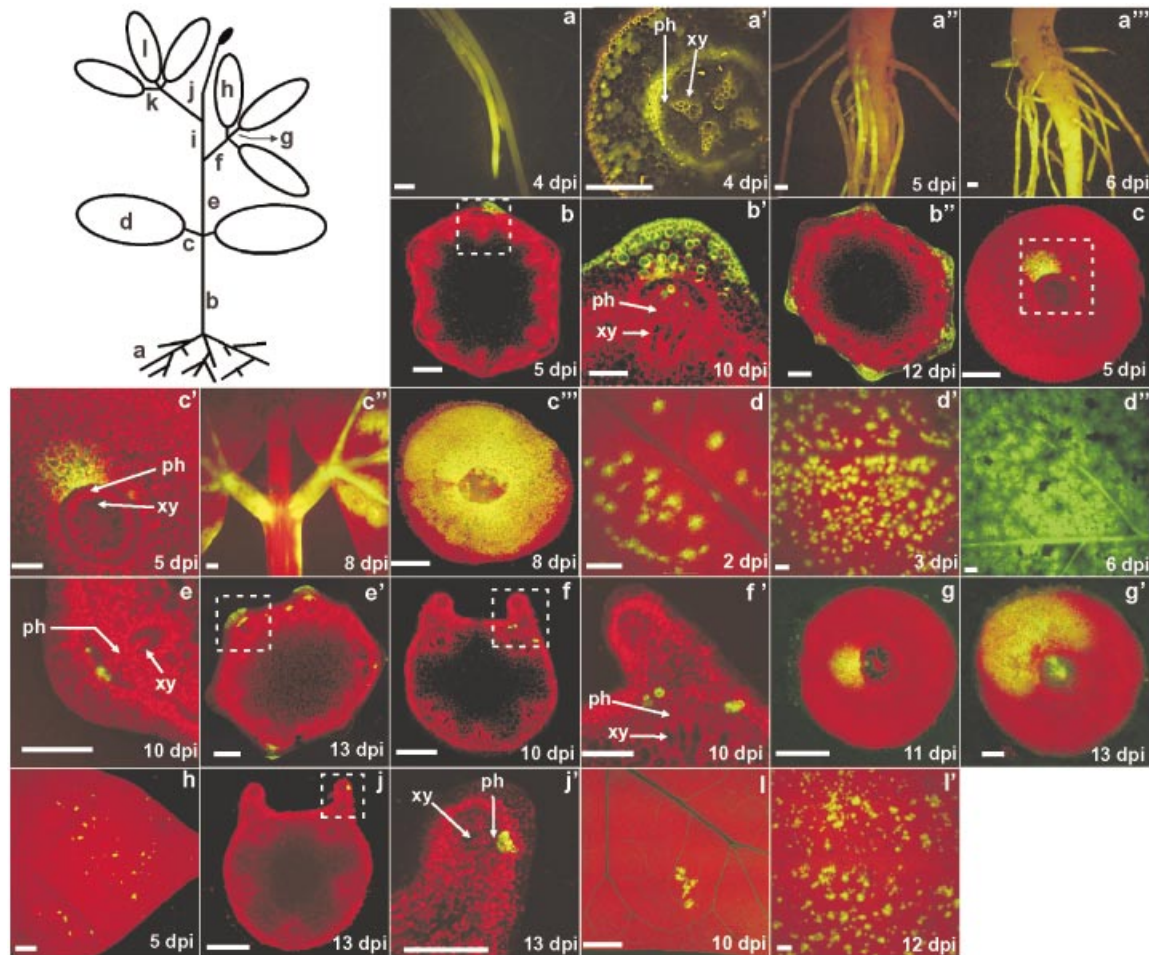


Fig. 2. Kinetics of CPMV systemic movement in cowpea plants. Top left corner, schematic representation of a cowpea plant. Fluorescence images depict representative images of CPMV M19GFP7 infection in cowpea over time from 1 to 14 days p.i. Letter identification of fluorescence images corresponds to plant parts shown in the cowpea plant schematic representation and Table 1. Ph, phloem; Xy, xylem. Insets in (b), (c), (e), (f) and (j) are depicted in (b'), (c'), (e'), (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.

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 at a developmental stage permissive for virus unloading in the first trifoliolate leaf and leaf lamina

flaps/midveins were removed 4 days p.i. when the virus had entered the stem. The plants were screened for systemic infection of first trifoliolate leaves at 14 days p.i. For each plant, 5 to 10 leaf lamina flaps were inoculated with M19GFP7. After removing the inoculated flaps (4 days p.i.), each flap was examined 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 II 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 summarized 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

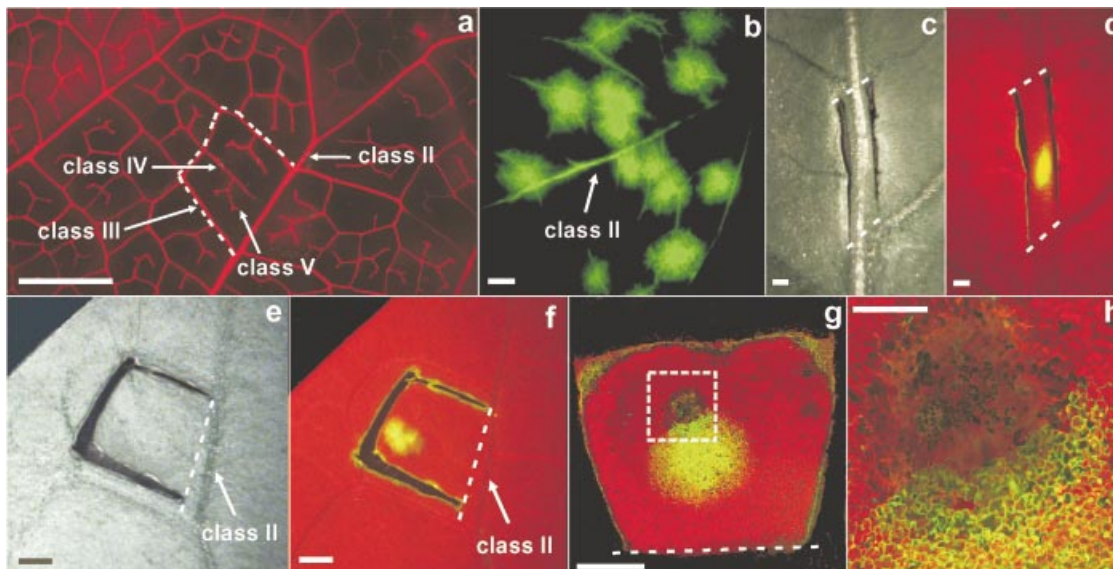


Fig. 3. Surgical procedure for determination of loading sites of CPMV in source primary leaves. (a) Veinal structure of a cowpea primary leaf visualized by Texas Red fluorescence. (b) Primary leaf at 4 days after inoculation with recombinant CPMV M19GFP7. (c, d) Brightfield and fluorescent images, respectively, of a pinpoint-inoculated isolated midvein at 4 days post-pinpoint inoculation. (e, f) Brightfield and fluorescent images, 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.

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

* No. of plants successfully inoculated and included in the experiment per total no. of plants treated.

† No. of isolated leaf lamina flaps that showed infection foci after inoculation per total no. of lamina flaps inoculated.

‡ Sum of results from the two independent experiments.

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.

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 trifoliolate 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 trifoliolate leaves in relation to the veinal structure (Fig. 4a and 4b). At very late stages of CPMV unloading (over 11 days p.i.) foci were difficult to observe (Fig. 4c). Fluorescent foci were predominantly associated with class III veins and occasionally

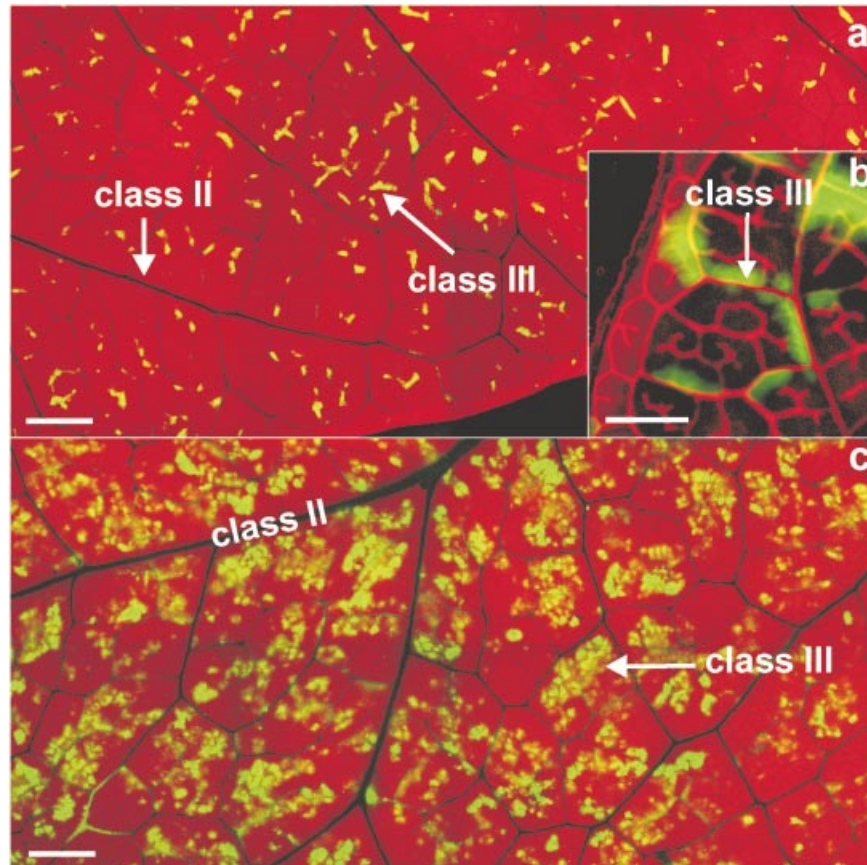


Fig. 4. CPMV unloading in the second sink trifoliolate leaves. Appearance of CPMV M19GFP2A-infected foci on trifoliolate cowpea leaves (a, b) at 5 days and (c) 11 days after inoculation of the primary leaves. (b) Inset shows fluorescent foci indicating unloading of virus from a class III vein. The large 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 trifoliolate leaves of M19GFP2A-inoculated cowpea plants at different days p.i.

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	

* No. of fluorescent foci recorded.

with class I and II veins, in particular at later stages of infection, but never with minor veins (Fig. 4b). Table 3 summarizes the distribution of a total of 2051 fluorescent foci in relation to the veinal structure, as recorded on trifoliolate 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

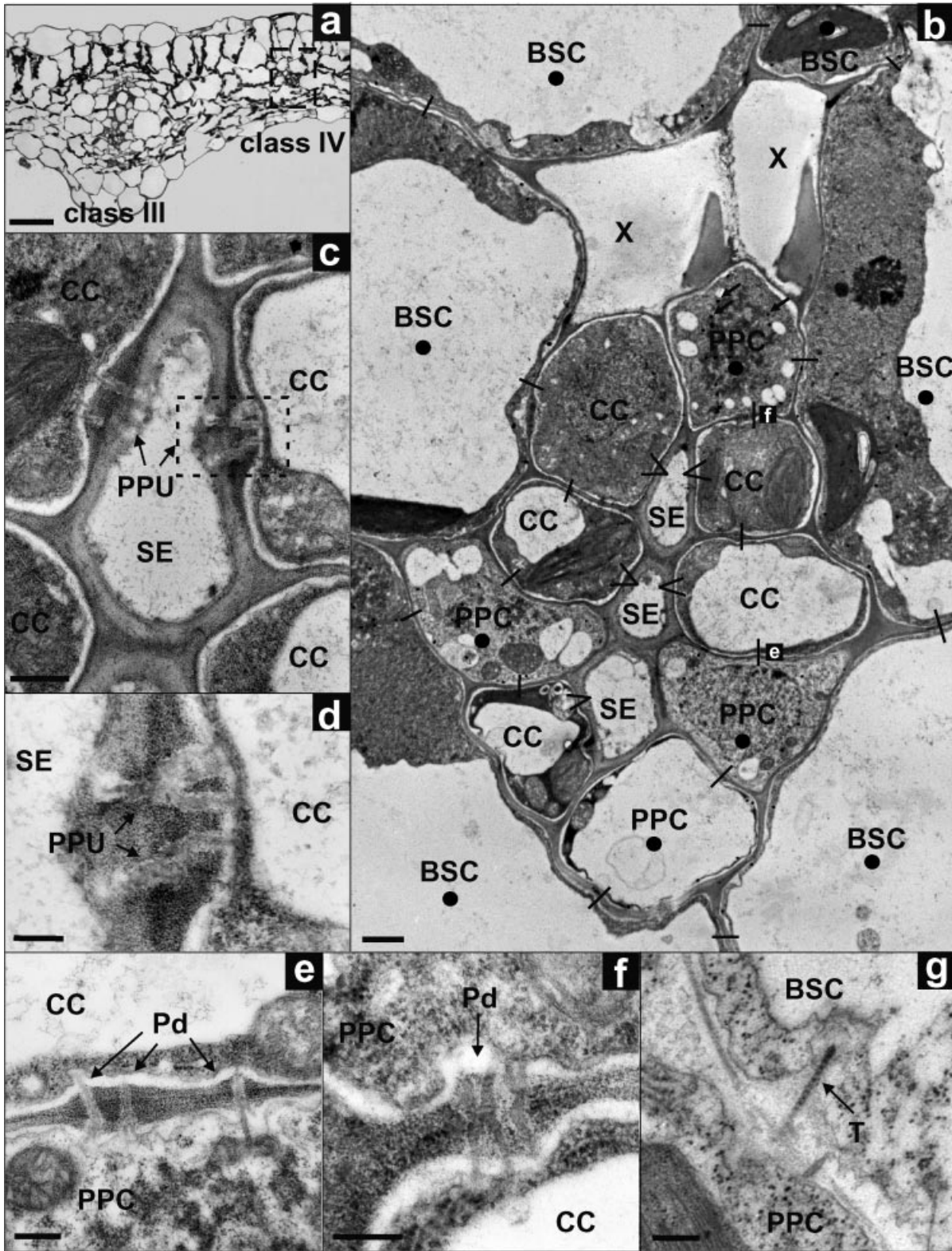


Fig. 5. CPMV vascular movement at cellular level. (a) Bright-field image of a cross-section of a primary leaf including a class III major and a class IV minor vein. (b) Corresponding EM image of the class IV vein in (a). The lines drawn through cell walls indicate the presence of linear or branched plasmodesmata (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.

trifoliolate leaves were analysed. Target veins were selected by the presence of associated fluorescent foci 3 days p.i. from inoculated leaves and 5 days p.i. from systemically infected trifoliolate leaves. In primary leaves, where the phloem loading occurred, class III and IV veins were excised for microscope analysis, while in systemically infected trifoliolate 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 CPs.

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 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. 5(a) and 5(b). In veins of both primary (source) and trifoliolate (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 cell types other than 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 CPs, 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 CP antigens were never detected in plasmodesmata at 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 over time 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 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 trifoliolate 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), the potyvirus *Tobacco etch virus* (TEV; Oparka & Santa Cruz, 2000) and the potyvirus *Potato X virus* (PVX; Roberts *et al.*, 1997) in *Nicotiana benthamiana*. Remarkably, a diverse range of phloem-transported com-

(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).

pounds 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 and 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 the tobamovirus *Sunm-hemp mosaic virus* (SHMV) in *Phaseolus vulgaris* and *Pisum sativum* (Ding *et al.*, 1998). For the potyviruses *Potato Y virus* (PVY) and *Peanut stripe virus* (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 the cucumovirus *Cucumber mosaic virus* (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 localization in unloading vascular tissue differs from that of PVX (Roberts *et al.*, 1997), BDMV (Wang *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 PPU was reported for the luteoviruses *Carrot red leaf virus* in *Anthriscus cerefolium* (Murant & Roberts, 1979) and *Potato leafroll virus* in potato (Shepardson *et al.*, 1980), and for the polerovirus *Beet western yellows virus* 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 PPU 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 PPU 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, remain to be determined.

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References

- Bendayan, M. & Zollinger, M. (1983).** Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A–gold technique. *Journal of Histochemistry and Cytochemistry* **31**, 101–109.
- Blackman, L. M., Boevink, P., Santa Cruz, S., Palukaitis, P. & Oparka, K. J. (1998).** The movement protein of cucumber mosaic virus traffics into sieve elements in minor veins of *Nicotiana clevelandii*. *Plant Cell* **10**, 525–537.

- Canto, T. & Palukaitis, P. (1999).** Are tubules generated by the 3a protein necessary for cucumber mosaic virus movement? *Molecular Plant–Microbe Interactions* **12**, 985–993.
- Carrington, J. C., Kasschau, K. D., Mahajan, S. K. & Schaad, M. C. (1996).** Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* **8**, 1669–1681.
- Chapman, S., Kavanagh, T. & Baulcombe, D. (1992).** Potato virus X as a vector for the gene expression in plants. *Plant Journal* **2**, 549–557.
- Cheng, N. H., Su, C. L., Carter, S. A. & Nelson, R. S. (2000).** Vascular invasion routes and systemic accumulation patterns of tobacco mosaic virus in *Nicotiana benthamiana*. *Plant Journal* **23**, 349–362.
- D’Arcy, C. J. & Zoeten, G. A. (1979).** Beet western yellows virus in phloem tissue of *Thlaspi arvense*. *Phytopathology* **69**, 1194–1198.
- Ding, X. S., Carter, S. A., Deom, C. M. & Nelson, R. S. (1998).** Tobamovirus and potyvirus accumulation in minor veins of inoculated leaves from representatives of Solanaceae and Fabaceae. *Plant Physiology* **116**, 125–136.
- Esau, K. & Hoefert, L. (1972).** Development of infection with beet western yellows virus in sugarbeet. *Virology* **48**, 724–738.
- Fisher, D. B. & Cash-Clark, C. E. (2000).** Sieve tube unloading and post-phloem transport of fluorescent tracers and proteins injected into sieve tubes via severed aphid stylets. *Plant Physiology* **123**, 125–137.
- Foster, R. L. S., Beck, D. L., Guilford, P. J., Voot, D. M., Van Dolleweerd, C. J. & Andersen, M. T. (1992).** The coat protein of white clover mosaic potyvirus has a role in facilitating cell-to-cell transport in plants. *Virology* **191**, 480–484.
- Gilbertson, R. L. & Lucas, W. J. (1996).** How do viruses traffic on the ‘vascular highway’? *Trends in Plant Science* **1**, 260–268.
- Goldbach, R. W. & Wellink, J. (1996).** Comoviruses: molecular biology and replication. In *The Plant Viruses: Polyhedral Virions and Bipartite RNA Genomes*, 1st edn, vol. 5, pp. 35–76. Edited by B. D. Harrison & A. F. Murant. New York: Plenum.
- Gopinath, K., Wellink, J., Porta, C., Taylor, K. M., Lomonossoff, G. P. & Van Kammen, A. (2000).** Engineering cowpea mosaic virus RNA-2 into a vector to express heterologous proteins in plants. *Virology* **267**, 159–173.
- Hibi, T., Rezelman, G. & Van Kammen, A. (1975).** Infection of cowpea mesophyll protoplasts with cowpea mosaic virus. *Virology* **64**, 308–318.
- Hickey, L. J. (1979).** A revised classification of the architecture of dicotyledonous leaves. In *Anatomy of the Dicotyledons*, pp. 25–39. Edited by C. R. Metcalf & L. Chalk. New York: Oxford University Press.
- Kasteel, D., Wellink, J., Verver, J., Van Lent, J., Goldbach, R. & Van Kammen, A. (1993).** The involvement of cowpea mosaic virus MRNA-encoded proteins in tubule formation. *Journal of General Virology* **74**, 1721–1724.
- Kempers, R. & Van Bel, A. J. E. (1997).** Symplasmic connections between sieve element and companion cell in the stem phloem of *Vicia faba* L. have a molecular exclusion limit of at least 10 kDa. *Planta* **201**, 195–201.
- Kempers, R., Prior, D. A. M., Van Bel, A. J. E. & Oparka, K. J. (1993).** Plasmodesmata between sieve element and companion cell of extrafascicular phloem of *Cucurbita maxima* permit passage of 3 kDa fluorescent probes. *Plant Journal* **4**, 567–575.
- Leisner, S. M., Turgeon, R. & Howell, S. H. (1992).** Long distance movement of cauliflower mosaic virus in infected turnip plants. *Molecular Plant–Microbe Interactions* **5**, 41–47.
- Leisner, S. M., Turgeon, R. & Howell, S. H. (1993).** Effects of host plant development and genetic determinants on the long-distance movement of cauliflower mosaic virus in *Arabidopsis*. *Plant Cell* **5**, 191–202.
- Murant, A. F. & Roberts, I. M. (1979).** Virus-like particles in phloem tissue of chervil (*Anthriscus cerefolium*) infected with carrot red leaf virus. *Annals of Applied Biology* **92**, 343–346.
- Mutterer, J. D., Stussi-Garaud, C., Michler, P., Richards, K. E., Jonard, G. & Ziegler-Graff, V. (1999).** Role of the beet western yellows virus readthrough protein in virus movement in *Nicotiana clevelandii*. *Journal of General Virology* **80**, 2771–2778.
- Nelson, R. S. & Van Bel, A. J. E. (1998).** The mystery of virus trafficking into, through and out of vascular tissue. *Progress in Botany* **59**, 476–533.
- Oparka, K. J. & Santa Cruz, S. (2000).** The great escape: phloem transport and unloading of macromolecules. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 323–347.
- Oparka, K. J., Roberts, A. G., Prior, D. A. M. & Santa Cruz, S. (1996).** Viral coat protein is targeted to, but does not gate, plasmodesmata during cell-to-cell movement of potato virus X. *Plant Journal* **10**, 805–813.
- Perbal, M. C., Thomas, C. L. & Maule, A. J. (1993).** Cauliflower mosaic virus gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. *Virology* **195**, 281–285.
- Reynolds, E. S. (1963).** The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.
- Ritzenthaler, C., Schmit, A. C., Michler, P., Stussi-Garaud, C. & Pinck, L. (1995).** Grapevine fanleaf nepovirus P38 putative movement protein is located on tubules *in vivo*. *Molecular Plant–Microbe Interactions* **8**, 379–387.
- Roberts, A. G., Santa Cruz, S., Roberts, I. M., Prior, D. A. M. & Turgeon, R. (1997).** Phloem unloading in sink leaves of *Nicotiana benthamiana*: comparison of a fluorescent solute with a fluorescent virus. *Plant Cell* **9**, 1381–1396.
- Santa Cruz, S. (1999).** Perspective: phloem transport of viruses and macromolecules – what goes in must come out. *Trends in Microbiology* **7**, 237–241.
- Séron, K. & Haenni, A. L. (1996).** Vascular movement of plant viruses. *Molecular Plant–Microbe Interactions* **9**, 435–442.
- Shepardson, S., Esau, K. & McCrum, R. (1980).** Ultrastructure of potato leaf phloem infected with potato leafroll virus. *Virology* **105**, 379–392.
- Storms, M. M. H., Van der Schoot, C., Prins, M., Kormelink, R., Van Lent, J. W. M. & Goldbach, R. W. (1995).** The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. *Virology* **214**, 485–493.
- Sudarshana, M. R., Wang, H. L., Lucas, W. J. & Gilbertson, R. L. (1998).** Dynamics of bean dwarf mosaic geminivirus cell-to-cell and long-distance movement in *Phaseolus vulgaris* revealed, using the green fluorescent protein. *Molecular Plant–Microbe Interactions* **11**, 277–291.
- Thompson, G. A. & Schulz, A. (1999).** Macromolecular trafficking in the phloem. *Trends in Plant Science* **4**, 354–360.
- Turgeon, R. (2000).** Plasmodesmata and solute exchange in the phloem. *Australian Journal of Plant Physiology* **27**, 521–529.
- Van Beek, N. A. M., Derksen, A. C. G. & Dijkstra, J. (1985).** Polyethylene glycol-mediated infection of cowpea protoplasts with sonchus yellow net virus. *Journal of General Virology* **66**, 551–557.
- Van Bel, A. J. E. (1996).** Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons. *Journal of Experimental Botany* **47**, 1129–1140.
- Van Bel, A. J. E. & Kempers, R. (1997).** The pore/plasmodesma unit: key element in the interplay between sieve element and the companion cell. *Progress in Botany* **58**, 278–291.

- Van Bokhoven, H., Verver, J., Wellink, J. & Van Kammen, A. (1993).** Protoplasts transiently expressing the 200K coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA. *Journal of General Virology* **74**, 2233–2241.
- Van Lent, J. W. M. & Verduin, B. J. M. (1986).** Detection of viral protein and particles in thin sections of infected plant tissue using immunogold labelling. In *Developments in Applied Biology: Developments and Applications in Virus Testing*, 1st edn, vol. 1, pp. 193–211. Edited by R. A. C. Jones & L. Torrance. Wellesbourne: Association of Applied Biologists.
- Van Lent, J. W. M. & Verduin, B. J. M. (1987).** Detection of viral antigen in semi-thin sections of plant tissue by immunogold-silver staining and light microscopy. *Netherlands Journal of Pathology* **93**, 261–272.
- Van Lent, J., Storms, M. & Goldbach, R. (1990).** Evidence for the involvement of 58K and 48K proteins in the intercellular movement of cowpea mosaic virus. *Journal of General Virology* **71**, 219–223.
- Van Lent, J., Storms, M., Van Der Meer, F., Wellink, J. & Goldbach, R. (1991).** Tubular structures involved in movement of cowpea mosaic virus are also formed in infected protoplasts. *Journal of General Virology* **72**, 2615–2623.
- Wang, H. L., Gilbertson, R. L. & Lucas, W. J. (1996).** Spatial and temporal distribution of Bean dwarf mosaic geminivirus in *Phaseolus vulgaris* and *Nicotiana benthamiana*. *Phytopathology* **86**, 1204–1214.
- Wellink, J. & Van Kammen, A. B. (1989).** Cell-to-cell transport of cowpea mosaic virus requires both the 58K/48K proteins and the capsid proteins. *Journal of General Virology* **70**, 2219–2286.
- Wellink, J., Jaegle, M. & Goldbach, R. (1987a).** Detection of a novel protein encoded by the bottom-component RNA of cowpea mosaic virus, using antibodies raised against a synthetic peptide. *Journal of Virology* **61**, 236–238.
- Wellink, J., Jaegle, M., Prinz, H., Van Kammen, A. & Goldbach, R. (1987b).** Expression of the middle component RNA of cowpea mosaic virus *in vivo*. *Journal of General Virology* **68**, 2577–2585.
- Wieczorek, A. & Sanfaçon, H. (1993).** Characterization and subcellular location of tomato ringspot nepovirus putative movement protein. *Virology* **194**, 734–742.
- Wisniewski, L. A., Powell, P. A., Nelson, R. S. & Beachy, R. N. (1990).** Local and systemic spread of tobacco mosaic virus in transgenic tobacco. *Plant Cell* **2**, 559–567.

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