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# Analysis of the systemic colonization of cucumber plants by *Cucumber green mottle mosaic virus*

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Systemic movement of *Cucumber green mottle mosaic virus* (CGMMV) in cucumber plants was shown to be from photoassimilate source to sink, thus indicating phloem transport. Nevertheless, CGMMV was not detected by immunocytochemical procedures in the intermediary cell–sieve element complex in inoculated cotyledons, where photoassimilate loading occurs. In stem internodes, CGMMV was first localized in the companion cells of the external phloem and subsequently in all tissues except the medulla, therefore suggesting leakage of the virus from, and reloading into, the transport phloem during systemic movement. In systemically infected sink leaves, CGMMV was simultaneously detected in the xylem and phloem. Interestingly, CGMMV accumulated to high levels in the differentiating tracheids of young leaves implying that the xylem could be involved in the systemic movement of CGMMV. This possibility was tested using plants in which cell death was induced in a portion of the stem by steam treatment. At 24 °C, steam treatment effectively prevented the systemic movement of CGMMV, even though viral RNA was detected in washes of the xylem above the steamed internode suggesting that xylem circulation occurred. At 29 °C, CGMMV systemically infected steam-treated cucumber plants, indicating that CGMMV can move systemically via the xylem. Xylem transport of CGMMV was, however, less efficient than phloem transport in terms of the time required for systemic infection and the percentage of plants infected.

## INTRODUCTION

Systemic transport through the vascular system is a crucial step in plant virus infection. Most plant viruses are thought to move systemically through the phloem in parallel with photoassimilate transport (reviewed by Haywood *et al.*, 2002; Lucas & Wolf, 1999; Nelson & Van Bel, 1998; Oparka & Turgeon, 1999; Thompson & Schulz, 1999). Phloem transport includes the loading (entry) of the virus into the phloem at source tissues, its circulation in the transport phloem and its unloading (exit) from the phloem at the sink tissues. Photoassimilate loading occurs presumably in the minor veins of source organs (Nelson & Van Bel, 1998; Santa Cruz, 1999) and in the case of cucurbits is thought to follow a symplastic route involving the specialized intermediary cell–sieve element complex (Turgeon, 1996). Knowledge of phloem circulation of plant viruses is far from complete, yet it is known that it parallels photoassimilate flow (Leisner *et al.*, 1992; Oparka & Turgeon, 1999). The requirement of a functional capsid protein (CP) for systemic movement is common but not universal, depending on specific virus–host interactions (e.g. Dalmay *et al.*, 1992; McGeachy & Barker, 2000; Petty & Jackson, 1990; Ryabov *et al.*, 2001), and for most viruses the precise form in which the virus is transported has not been determined. Phloem unloading in the sink organs also parallels photoassimilate unloading and proceeds basipetally in the developing leaf during the sink-to-source transition (Cheng *et al.*, 2000; Leisner *et al.*, 1992; Roberts *et al.*, 1997; Santa Cruz *et al.*, 1998).

While it is assumed that most viruses move systemically through the phloem, xylem transport has been proposed for some viruses, most notably for members of the beetle-transmitted *Sobemovirus* genus. Although not a clear indicator of a role for xylem in systemic movement, localization of virus particles in the xylem has been reported in many instances (e.g. Dubois *et al.*, 1994; Fribourg *et al.*, 1987; Khan *et al.*, 1994; Urban *et al.*, 1989; Verchot *et al.*, 2001). Further evidence supporting xylem transport has been reported in a few instances (Dubois *et al.*, 1994; Opalka *et al.*, 2000; Schneider & Worley, 1959a, b). As well as the growing number of reported cases, one aspect of virus systemic movement via the xylem that still needs to be addressed is exactly how viruses translocate from the non-living tracheary elements to living cells (i.e. from the apoplast to the symplast) for the establishment of systemic infection. To explain this, a model by which the virus chelates calcium in the pit membrane between tracheids,

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relaxing the membrane's structure, and then moves into living immature tracheids has been proposed for *Rice yellow mottle virus* (Opalka *et al.*, 2000). Hence, systemic movement of plant viruses through the xylem is a relatively unexplored process. Further studies are needed for a better knowledge of the occurrence of xylem transport in different viral taxa and to analyse the mechanism of xylem transport of plant viruses and its relevance compared with systemic movement through the phloem.

*Cucumber green mottle mosaic virus* (CGMMV) is a *Tobamovirus* that shares the same genetic organization and similar particle morphology as *Tobacco mosaic virus* (TMV) (Fukuda *et al.*, 1981; Namba *et al.*, 1989; Ugaki *et al.*, 1991; Wang & Stubbs, 1994). The systemic transport of CGMMV has been analysed in our laboratory and was shown to circulate as virus particles in the phloem of infected cucumber plants (*Cucumis sativus* L.) (Simón-Buela & García-Arenal, 1999). In this work, we report on the infection progress and cellular localization of CGMMV during the systemic colonization of cucumber plants. The data support previous results on the role of phloem in the systemic transport of CGMMV and show that CGMMV can also move systemically through the xylem. Both types of vascular tissue were used by CGMMV for systemic infection of cucumber plants, but systemic movement through the phloem was shown to be a more efficient process.

## METHODS

**Viruses and plants.** CGMMV strain SH (Ugaki *et al.*, 1991) was grown in cucumber plants in the greenhouse and purified according to Fukuda *et al.* (1981). Plants were inoculated in the adaxial surface of cotyledons or leaves with CGMMV particles at a final concentration of 300 μg $ml^{-1}$ in 0·1 M $Na_2HPO_4$, pH 7·0. For immunolocalization analyses, the cotyledons of plants in which the first true leaf was expanding and was about 5 mm long (approximately 10 days after sowing) were inoculated with 10 μl of inoculum. For the analysis of CGMMV systemic movement in steamed plants (see below), cucumber plants with three expanded leaves were inoculated in the cotyledons and in the first true leaf with 30 μl of inoculum. Unless specified, inoculated and mock-inoculated control plants were maintained in growth chambers at 24 °C with a 16 h light/8 h dark cycle and 70 % humidity. *Cucumber mosaic virus* (CMV, *Cucumovirus*) strain *Fny* was obtained from biologically active clones (Rizzo & Palukaitis, 1990) and multiplied in *Nicotiana tabacum* cv. Xanthi nc. CMV RNA was purified from infected tobacco plants and inoculated as described by Thompson & García-Arenal (1998).

**Microscopy.** Samples of plant material taken from CGMMV-inoculated plants at 4, 8, 12, 16, 20 and 24 days post-inoculation (p.i.) were cut to the approximate dimensions of 1 × 1 × 4 mm and fixed and embedded as described by Thompson & García-Arenal (1998). This material consisted of the following plant parts: the inoculated cotyledon, every leaf in the plant [one (1st) at 4 days p.i. and five (1st–5th) by 20–24 days p.i.] and stem internodes [one (1st) at 8 days p.i. and four (1st–4th) by 24 days p.i.] (Table 1). The youngest analysed leaf of the plant was at least 4 mm in size. The most apical internode analysed was the one below the youngest analysed leaf (for example, at 20 days p.i. the 4th internode, below the 5th leaf; Table 1). Samples were taken from the central region of the leaf longitudinally following the path of veins of order I, II and III. Internode samples were taken longitudinally from a portion equidistant from each node. Similar samples were taken from mock-inoculated controls at 12 and 24 days p.i. Immunolocalization in semi- (1 μm) and ultrathin (100–140 nm) sections using light and electron microscopy, respectively, was carried out according to the protocols described by Thompson & García-Arenal (1998). All sections were cut with glass knives with a Reichert ultramicrotome. Light microscopy was carried out with a Zeiss Axiophot and electron microscopy with a JEOL model JEM-1200EX II.

**Table 1.** Detection of CGMMV infection in various organs of infected cucumber plants

Data are percentage of samples positive for immunosilver CP detection in semi-thin sections for *N* analysed specimens at various days p.i. Leaves and stem internodes are relative to the inoculated cotyledons.

| Plant organ | Days p.i. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | | 8 | | 12 | | 16 | | 20 | | 24 | |
| | *N* | % | *N* | % | *N* | % | *N* | % | *N* | % | *N* | % |
| Cotyledon | 9 | 33 | 9 | 33 | 9 | 33 | 9 | 33 | 14 | 43 | 8 | 38 |
| 1st leaf | 9 | 0 | 12 | 0 | 13 | 0 | 9 | 11 | 6 | 0 | – | – |
| 1st internode | | | 12 | 0 | 13 | 15 | – | – | – | – | 6 | 33 |
| 2nd leaf | | | 12 | 0 | 13 | 0 | 9 | 44 | 8 | 25 | – | – |
| 2nd internode | | | | | 13 | 7 | 8 | 25 | 11 | 36 | 11 | 64 |
| 3rd leaf | | | | | 14 | 14 | 13 | 30 | 6 | 83 | – | – |
| 3rd internode | | | | | | | 14 | 43 | 9 | 66 | 11 | 64 |
| 4th leaf | | | | | | | 14 | 21 | 11 | 82 | 5 | 100 |
| 4th internode | | | | | | | | | 11 | 11 | 14 | 29 |
| 5th leaf | | | | | | | | | 9 | 55 | 9 | 67 |

**Western immunoblots.** Leaf lamina, root tip, xylem wash or phloem exudate samples were homogenized in 1 vol. 0·2 M Tris/HCl, pH 8·2, 2 mM EDTA, 0·1 % SDS, incubated at 70 °C for 10 min and centrifuged at 10 000 ***g*** to pellet the debris. Leaf and root samples corresponded to 0·1 g fresh tissue. Xylem washes were obtained from an excised stem segment about 20 cm long in which petioles were cut as described by Chambers & Francki (1966). The bottom part of the stem (2 cm) was immersed for 2 min in boiling water and the stem was washed under gentle vacuum with 20 μl drops of 0·1 M $Na_2HPO_4$, pH 7·0, applied to the top. The wash was collected in a 10 ml tube placed in the vacuum chamber. After each drop application and suction, 0·5 cm stem segments were removed before applying the following drop. In total, about 200 μl of xylem wash was collected from each stem and concentrated by lyophylization. Phloem exudate was obtained as described by Simón-Buela & García-Arenal (1999). Extracts corresponding to 5 mg of leaf or root tissue, to 10 μl of phloem exudate or to 100 μl of xylem wash were electrophoresed by 15 % SDS-PAGE and electrotransferred to nitrocellulose membranes as described by Sambrook *et al.* (1989). Immunoblots and quantification of CGMMV in immunoblots were carried out as described (Moreno *et al.*, 1997) using an antiserum against CGMMV (Simón-Buela & García-Arenal, 1999).

**Nucleic acid extraction and analyses.** Nucleic acids were extracted from 0·2 g fresh weight cotyledon samples, 0·1 g fresh weight leaf and root samples, 10 μl phloem exudate and 100 μl xylem washes. These samples were homogenized in 100 μl 0·2 M Tris/HCl, pH 8·2, 2 mM EDTA, 0·1 % SDS, incubated for 10 min at 70 °C and extracted in 2 : 1 vols of phenol : chloroform (1 : 1 by volume). Nucleic acids were precipitated at 4 °C in 2 M LiCl and concentrated by centrifugation at 10 000 ***g*** for 15 min. The nucleic

acid pellets were washed with 200 µl 70 % ethanol and resuspended in 100 µl (leaf or root extracts) or 10 µl (phloem or xylem extracts) of double-distilled sterile water.

For Northern blot hybridization analyses, nucleic acid extracts corresponded to 20 mg fresh leaf or root tissue, 10 µl phloem exudate or 100 µl total xylem wash. The extracts were denatured as described by Sambrook *et al.* (1989) and blotted onto Hybond-N membranes (Amersham) using a manifold apparatus (Bio-Rad). CGMMV RNA was detected with a $^{32}$P-labelled RNA probe complementary to nt 4322–4681 of CGMMV-SH RNA. The lower limit for RNA detection by this procedure was about 1·5 ng as estimated from known amounts of CGMMV RNA diluted in a fivefold series in nucleic acid extract from uninfected plants (not shown). For CMV-Fny RNA detection, a $^{32}$P-labelled RNA probe complementary to the CP gene (Fraile *et al.*, 1997) was used.

For RT-PCR detection of CGMMV RNA, nucleic acid extracts corresponding to 4 mg fresh leaf or root tissue, 1 µl phloem sap or 10 µl total xylem wash were used. A 359 nt fragment representing nt 4322–4681 of CGMMV-SH was amplified using oligonucleotides LS1 (5′-GTTTCGCCTCAAAATTCC-3′) and LS2 (5′-TCTAAATAT-GACAAGTCGC-3′) as described by Simón-Buela & García-Arenal (1999). The lower limit for CGMMV RNA detection was about 8 pg as estimated from known amounts of CGMMV RNA diluted in a fivefold series in nucleic acid extracts from uninfected plants (not shown).

**Protoplast isolation and FITC-labelling.** Mesophyll protoplasts from cucumber cotyledons were obtained and isolated as described by Taliansky & García-Arenal (1995). Each sample consisted of a pool of six cotyledon halves excised from inoculated plants or from uninoculated controls. After isolation, mesophyll protoplasts were fixed for 15 min in 10 ml 95 % ethanol and washed three times in 10 ml 10 mM Tris/HCl, pH 7·5, 100 mM NaCl. Protoplasts were then incubated for 2 h at 37 °C in 500 µl 10 mM Tris/HCl, pH 7·5, 100 mM NaCl, 1 % BSA with a 1 : 100 dilution of CGMMV antiserum (Simón-Buela & García-Arenal, 1999). Protoplasts were washed as described before and incubated for 2 h at 37 °C in 500 µl 10 mM Tris/HCl, pH 7·5, 100 mM NaCl, 1 % BSA, with a 1 : 160 dilution of FITC-conjugate anti-rabbit IgG (Sigma F9887). After three additional washes, protoplasts were counted under a light microscope using a haemocytometer grid. FITC-labelled protoplasts were counted using a 450–490 nm filter in a Zeiss Axiophot.

## RESULTS

### Progress of CGMMV infection in cucumber plants

The colonization of cucumber plants by CGMMV was analysed by immunochemical and immunocytochemical detection of the viral CP in different plant organs on different days p.i. Leaves and stem internodes, numbered according to their position relative to the inoculated cotyledons, were analysed. The first true leaf (1st leaf) and the cotyledons were very close to each other with no appreciable internode between them. We numbered as 1st internode the one above the 1st leaf and below the 2nd leaf, the 2nd internode separated the 2nd and 3rd leaves, and so on. Immunocytochemical detection of CGMMV in semi-thin sections of 354 cotyledon, leaf or internode samples was scored and classified according to the time of collection and the source organ (Table 1). Results showed that, in inoculated cotyledons, the percentage of positive samples did not significantly increase ($\chi^2$ test) after 4 days p.i. (Table 1). Similarly, the amount of CP detected by Western immunoblot did not increase after 8 days p.i. (not shown). Systemic infection was first detected at 12 days p.i. in the 1st and 2nd internodes and in the youngest analysed leaf (3rd leaf) indicating that young expanding leaves are the first to become systemically infected (Table 1). Western immunoblots detected systemic infection only after 16 days p.i. (not shown). Infection of the 1st and 2nd leaves at 16 days p.i. could be the result of long-distance or cell-to-cell movement. Typically, the percentage of positive samples in young expanding leaves increased with time and, for each leaf above the 2nd leaf, the percentage of positive samples also increased with time. The observed progress of CGMMV infection was consistent with sink-to-source phloem transport to developing leaves. In addition, CGMMV colonized fully expanded mature leaves that might already have undergone the sink-to-source transition.

### Tissue and cellular localization of CGMMV

The progress of CGMMV infection in cucumber plants was analysed at the tissue and cellular level by immunosilver CP detection in semi-thin sections and immunogold CP detection in ultrathin sections. In the minor veins (order VII to V) of inoculated cotyledons, both the bundle sheath (BS) and the vascular parenchyma (VP) cells were shown to be infected at 8 days p.i. in 9 out of 17 veins scored, as exemplified in Fig. 1 (A) and (B). From 8 to 20 days p.i., high accumulation in xylem-associated VP was noteworthy (Fig. 1A). CGMMV was detected in the phloem intermediary cells (ICs) in only one out of nine minor veins showing infection of cells of the BS and VP (not shown). The VP–IC interface was observed as a boundary for CGMMV localization, as immunogold labelling of the CP was intense in the VP and completely lacking in the ICs (Fig. 2A and B). CGMMV was not detected in the sieve elements (SEs) of minor or major veins in the inoculated cotyledons. In the major veins (order III and IV) of cotyledons, CGMMV was localized in the BS (six out of seven observations) and the VP (seven out of seven observations), but not in the phloem. The high frequency of CGMMV infection in major veins suggests that virus loading may occur in major veins as well as in minor veins.

In the youngest infected internode, CGMMV was initially and frequently (in 9 out of 17 of the youngest internodes analysed) detected in the companion cells (CCs) of the external phloem, from which virus infection extended into the cortical parenchyma (Fig. 1D). In later stages, CGMMV infected the stem schlerenchyma and all stem tissues except the medullar parenchyma (not shown). Electron microscopy analyses showed CGMMV accumulation in the CCs but not in the SEs (Fig. 2E). Thus, in the transport phloem, the CCs of the external phloem are sites for CGMMV unloading for the colonization of stem tissues.

In the major veins (order I to III) of systemically infected

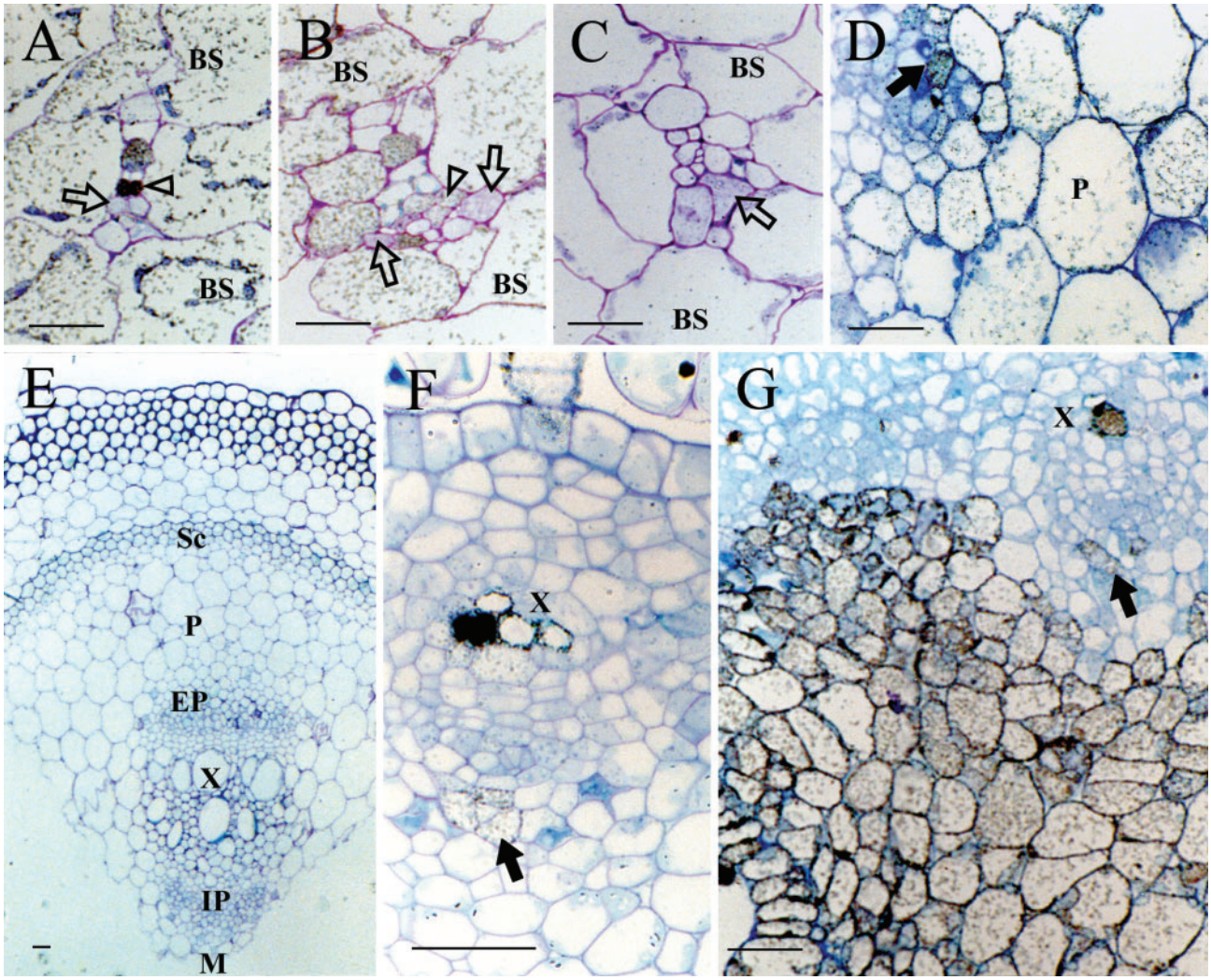


**Fig. 1.** Immunosilver detection of CGMMV in semi-thin sections of inoculated cucumber plants. (A, B) Minor veins from the inoculated cotyledons showing virus detection in bundle sheath cells (BS) and vascular parenchyma (open arrowheads), but not in intermediary cells (open arrows). (C) Minor vein from an uninoculated cotyledon. (D) Transverse section of the youngest stem internode showing CGMMV localization in the external phloem companion cells (black arrow) and in the cortex parenchyma (P). (E) Transverse section of the stem internode from a healthy plant stained with toluidine blue in which the cortex parenchyma (P), sclerenchyma (Sc), external phloem (EP), internal phloem (IP), xylem (X) and medullar parenchyma (M) are indicated. (F, G) Sections of young developing leaves, showing virus accumulation in xylem tracheids and associated parenchyma (X) and in the external phloem companion cells (black arrow), which in (G) are seen in the vicinity of an infection front from another vein. Bar, 20 μm.

young expanding leaves, CGMMV was first detected in the xylem cells, including the circulating elements, and in abaxial phloem CCs (arrows in Fig. 1F and G). Later on, high virus accumulation was observed in leaf parenchyma cells (Fig. 1G). Remarkably, CGMMV accumulated to high levels in xylem cells (Fig. 1F). Electron microscopy analysis showed that differentiating tracheary elements at different developmental stages were heavily labelled with immunogold (Fig. 2F–H), and large aggregates of immunogold-labelled virus-like particles were observed in some developing tracheary elements (T2 in Fig. 2F, magnified in Fig. 2G). Also, labelled virus-like particles were abundant on both sides of the middle lamella between tracheids (Fig. 2G). In mature systemically infected leaves, CGMMV was detected in all vascular cell types of minor veins, e.g. of 14 observed veins from the 2nd leaf at 16 days p.i., CGMMV was detected within the BS, VP and ICs in seven veins and in the SEs in five veins (not shown).

All the results presented in this section indicate long-distance transport of CGMMV through the phloem. In addition, the results show a marked tropism of CGMMV for the VP and xylem, suggestive of a role for this tissue in the long-distance transport of the virus.

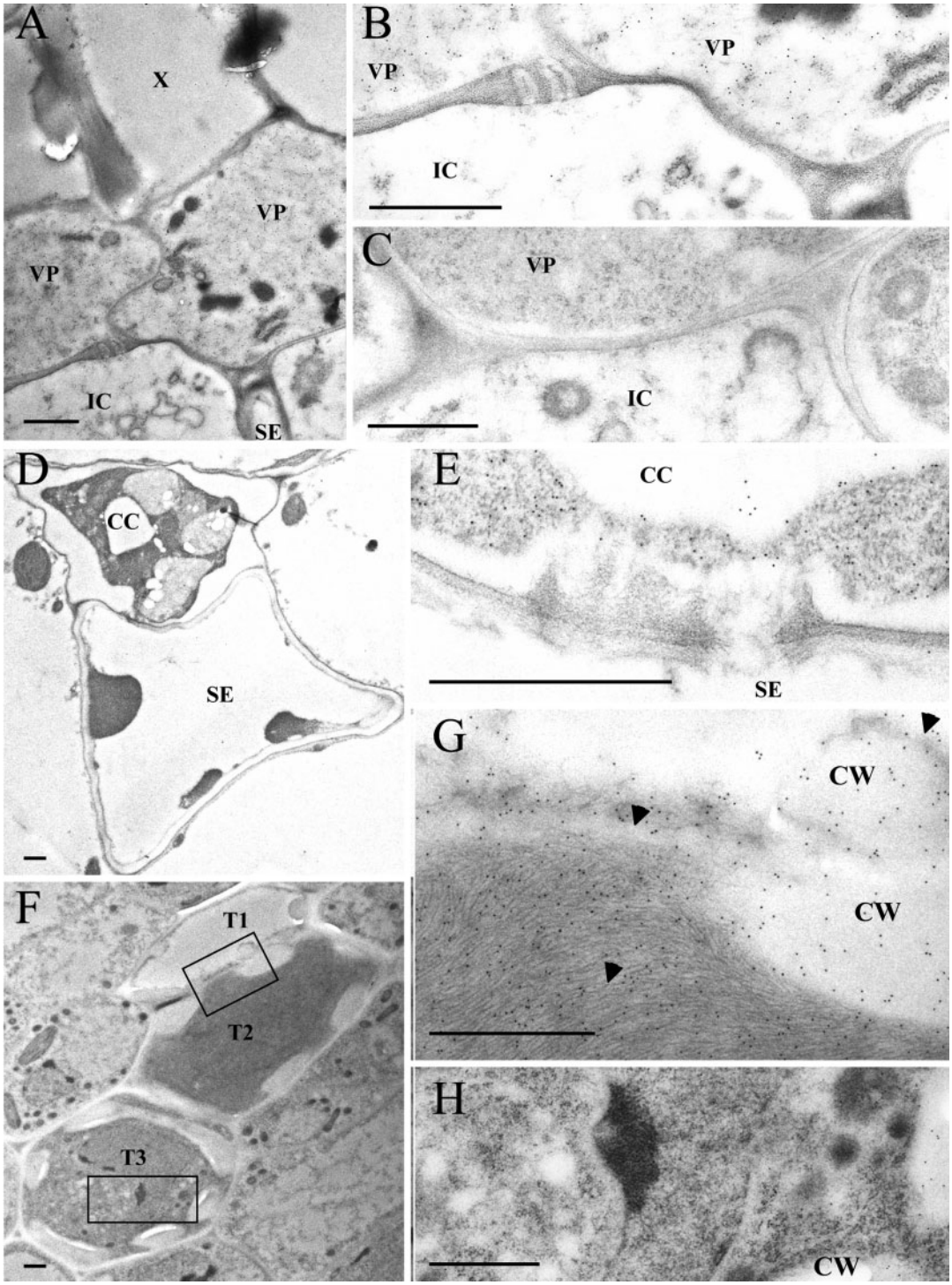


**Fig. 2.** Immunogold detection of CGMMV in ultrathin transverse sections of inoculated cucumber plants. (A) Minor vein in inoculated cotyledon showing xylem tracheary elements (X), vascular parenchyma cells (VP), intermediary cells (IC) and a sieve element (SE). (B) Magnification of (A) showing heavily gold-labelled VP cells and unlabelled IC. (C) Detail of a minor vein from an uninoculated cotyledon showing the VP–IC interface. (D) Transverse section of the stem internode from an uninoculated plant showing a companion cell (CC)–SE complex in the external phloem. (E) Detail of the CC–SE interface from the youngest stem internode showing plasmodesmal connections. Note that immunogold labelling is restricted to the CC. (F) Major vein from a young developing leaf showing tracheids in different developmental stages, labelled T1, T2 and T3. (G) Magnification of the cell lumen of T2 and the middle lamella between T1 and T2, showing immunogold labelling of virus-like aggregates in the T2 lumen, in both sides of the middle lamella and in the secondary walls (CW) (arrowheads). (H) Magnification of T3 showing less-dense immunogold labelling than in T2. Bar, 1 μm.

**Fig. 3.** Detail of a cucumber plant showing the effect of steam treatment in the first internode above the inoculated cotyledons 24 h after steaming. A segment of about 2 cm in length, reduced to a thin necrotic strand, is visible close to the prop.

## Assay of xylem and phloem transport of CGMMV

The role of phloem or xylem in the systemic transport of CGMMV was examined by analysing the progress of infection in plants in which cell death was induced in a portion of the 1st internode with a jet of steam (Schneider & Worley, 1959a). Plants with three fully expanded leaves and a well-developed 1st internode (10–15 cm long) were inoculated in the cotyledons and 1st leaf. Thereafter, a jet of steam was applied to the central portion of the 1st internode for about 15 s, until water soaking became apparent. One hour after steaming, a segment about 2 cm long was reduced to a very thin strand and showed dry necrosis by 1–2 days p.i. (Fig. 3). CMV, which has been used in the past as a negative control for xylem transport (Caldwell, 1930), was included in some experiments to test how efficiently phloem transport was prevented in steamed plants. Most steamed plants developed axillary shoots below the steamed internode 7 days p.i. From 7 days p.i. onwards, occasional death of plant parts above the steamed portion was observed, reducing the number of plants available for systemic movement analysis. Typically, more than 50 % of the steamed plants survived until 14 days p.i., although less than 20 % survived until 21 days p.i.

Systemic movement in steamed and in control plants was evaluated by symptom development in the leaves above the steamed internode, by Western immunochemical detection of CGMMV CP and by Northern blot hybridization detection of CGMMV RNA. The axillary shoots that grew below the steamed portion were also analysed. Initially (for example, in the experiments in Table 2) infection in the inoculated leaf and cotyledons was also analysed by Western blots and Northern blot hybridization. Results of CGMMV or CMV detection by Northern blot hybridization analyses from six independent assays are shown in Table 2 and indicated that only non-steamed plants were systemically infected with either virus above the 1st internode. RNA detection in axillary shoots indicated that systemic movement below the steamed internode was not inhibited by the treatment (Table 2). Data from CP detection or symptom development (not shown) were the same as those shown for RNA detection. Thus, it was concluded that both CGMMV and CMV moved systemically through the phloem.

Immunolocalization of CGMMV in the xylem of systemically infected cucumber plants indicated the presence of CGMMV in xylem elements (see previous section), which could result in its circulation across steamed portions. To examine this possibility, steamed plants inoculated with CGMMV and non-steamed controls were analysed 21 days p.i. No samples from these plants were gathered previous to this time, but at 21 days p.i. the plants were harvested and xylem washes, leaf and root samples and phloem

**Table 2.** Analysis of CMV and CGMMV infection in steamed cucumber plants

Data are percentages for *N* analysed plants in which CGMMV RNA was detected by Northern blot hybridization at 7 days p.i. for inoculated cotyledon and leaf samples, at 14 days p.i. for upper leaf or axillary shoot samples and at 21 days p.i. for upper leaf samples. Data were pooled from six different experiments. Treatments were CMV and CGMMV inoculations in steamed plants and in non-steamed controls.

| Treatment | Inoculated leaf | | Upper leaves | | | | Axillary shoots | |
|---|---|---|---|---|---|---|---|---|
| | 7 days p.i. | | 14 days p.i. | | 21 days p.i. | | 14 days p.i. | |
| | *N* | % Infected | *N* | % Infected | *N* | % Infected | *N* | % Infected |
| CMV | 14 | 93 | 14 | 100 | – | – | – | – |
| CMV steamed | 41 | 90 | 41 | 0 | – | – | 41 | 59 |
| CGMMV | 55 | 73 | 48 | 60 | 12 | 100 | – | – |
| CGMMV steamed | 133 | 79 | 116 | 0 | 16 | 0 | 47 | 72 |

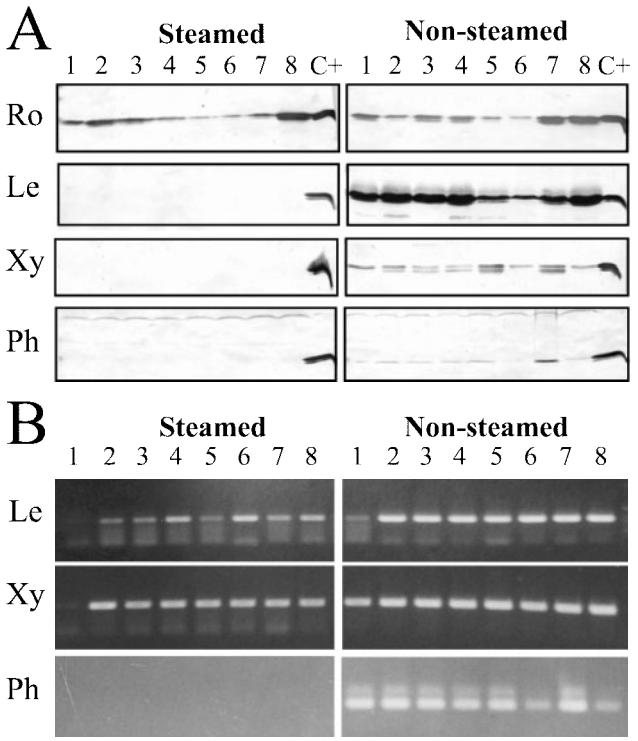


**Fig. 4.** CGMMV CP and RNA detection at 21 days p.i. in eight steamed cucumber plants and eight non-steamed controls. (A) Western immunodetection of CP in roots (Ro) and in the youngest leaf (Le), xylem wash (Xy) or phloem exudate (Ph) sampled above the 1st stem internode. Lanes 1–8 correspond to the eight different plants tested; lane C+ corresponds to 1 μg purified CGMMV virions. (B) Detection of CGMMV RNA by RT-PCR amplification of nt 4322–4681 in nucleic acid extracts from the youngest leaf (Le), xylem wash (Xy) or phloem exudate (Ph). Lanes 1–8 correspond to the same plants tested in (A).

exudates were all analysed for the presence of CGMMV CP and RNA. CGMMV CP was detected by Western immunoblot in root, leaf, xylem wash and phloem sap samples in non-steamed plants but only in root samples in steamed plants (Fig. 4A). Northern blot hybridization analysis gave the same results (not shown). However, when the presence of CGMMV RNA in these plant parts was analysed by RT-PCR, which was approximately 200 times more sensitive than Northern blot hybridization analysis (see Methods), the result was different. CGMMV RNA was detected in the leaves and xylem washes above the treated internode in seven out of eight analysed plants, but in no case was it detected in the phloem exudate (Fig. 4B). The possibility that RT-PCR detection of CGMMV RNA in xylem washes and in leaf extracts from steamed plants was the result of contamination could be discarded, as these plants were not manipulated prior to harvest and CGMMV RNA was not detected in phloem exudates. The results indicate that CGMMV RNA circulates and moves long distances through the xylem. However, under the conditions of this experiment, the presence of CGMMV RNA in the xylem above the steamed internode did not result in the systemic infection of, and symptom development in, the upper leaves.

As viruses cannot multiply in mature tracheary elements or in xylem vessels, it can be assumed that the amount of virus circulating in the xylem will depend on its access to this tissue, which in turn is related to the efficiency of virus accumulation and spread. Quantification of the percentage of infected mesophyll cells and of the CP accumulation at various times after inoculation indicated that cell-to-cell movement and virus accumulation in inoculated cotyledons were more efficient at 29 °C than at 24 °C. The extent of CGMMV accumulation and spread by 3–4 days p.i. at 29 °C was comparable with the observed values at 6–7 days p.i. at 24 °C (Fig. 5). Differences observed in the CP accumulation level and percentage of infected mesophyll cells at 6 days

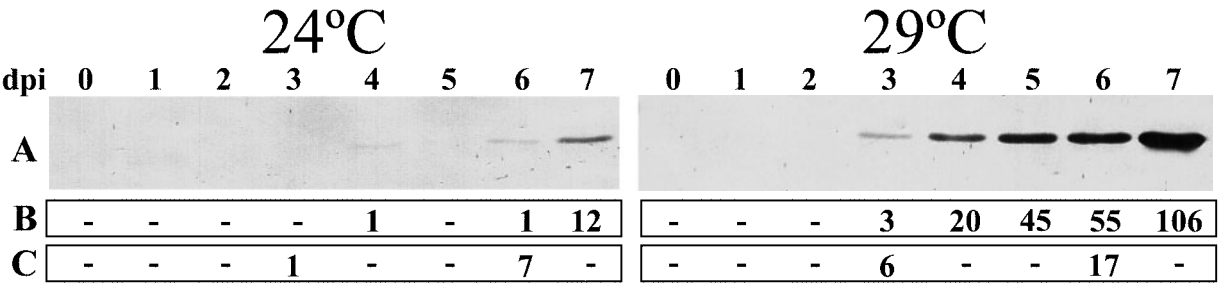


**Fig. 5.** Effect of temperature on the colonization of cucumber cotyledons by CGMMV. The numbers above the lanes indicate days p.i. (A) Western immunoblot analysis of the CP accumulation kinetics at 24 or 29 °C. Each sample consisted of six cotyledon disks of 5 mm in diameter taken from six different cotyledons at the indicated days p.i. and pooled prior to extraction and 15 % polyacrylamide/0·1 % SDS gel electrophoresis. (B) CP (μg) per gram of fresh tissue estimated from the lanes shown in (A) by interpolation of a standard curve ranging from 1 to 10 000 ng CGMMV virions serially diluted 1 : 2, electrophoresed and immunoblotted in parallel (not shown). (C) Percentage of isolated mesophyll protoplasts fluorescently labelled with the CGMMV CP antiserum and FITC-conjugated anti-rabbit IgG at 3 and 6 days p.i. Each sample corresponded to a pool of six cotyledon halves sampled and processed for protoplast isolation.

**Table 3.** Effect of temperature on the systemic infection in steamed or non-steamed cucumber plants inoculated with CGMMV

Data are percentages for *N* analysed plants in which CGMMV RNA was detected by Northern blot hybridization in upper leaf samples at 7 and 14 days p.i. Data are from two separate experiments. Treatments include CGMMV inoculations in steamed plants and non-steamed controls. Plants were grown at a constant temperature of either 24 or 29 °C.

| Experiment/ treatment | 7 Days p.i. | | | 14 Days p.i. | |
|---|---|---|---|---|---|
| | Temperature (°C) | *N* | % Infected | *N* | % Infected |
| **Expt I** | | | | | |
| CGMMV | 24 | 12 | 0 | 12 | 100 |
| CGMMV steamed | 24 | 45 | 0 | 25 | 0 |
| CGMMV | 29 | 22 | 100 | 22 | 100 |
| CGMMV steamed | 29 | 75 | 0 | 30 | 10·0 |
| **Expt II** | | | | | |
| CGMMV | 24 | 12 | 0 | 12 | 100 |
| CGMMV steamed | 24 | 45 | 0 | 30 | 0 |
| CGMMV | 29 | 12 | 100 | 12 | 100 |
| CGMMV steamed | 29 | 43 | 0 | 26 | 11·5 |

p.i. suggested that temperature affects both replication and cell-to-cell movement. These results prompted us to compare the systemic movement of CGMMV at 24 and 29 °C. Non-steamed inoculated controls were systemically infected by 7 days p.i. at 29 °C and by 12–14 days p.i. at 24 °C, in two different experiments (Table 3). All the plants in which systemic infection was detected by Northern blot hybridization analyses showed CGMMV symptoms (not shown). Thus, plant colonization was more efficient at 29 than at 24 °C. Regardless of the temperature conditions, all non-steamed plants became systemically infected (Table 3). Interestingly, CGMMV was detected in the upper leaves of about 11 % of steamed plants at 29 °C and these plants showed CGMMV symptoms by 12–14 days p.i. (not shown), whereas no steamed plant became systemically infected (Table 3) or showed CGMMV symptoms (not shown) at 24 °C. The fraction of steamed plants that became systemically infected at 29 °C was not affected by wounding of the inoculated leaves: in Experiment I in Table 3, samples from the inoculated leaf and cotyledons were taken for Northern blot hybridization analysis (not shown) at 7 days p.i., as well as samples from upper leaves. In Experiment II, only samples from the upper leaves were gathered and analysed at 7 days p.i. It is also worth noting that systemic infection of steamed plants was considerably delayed with respect to untreated controls (Table 3). These data show that transport through the xylem, although inefficient and dependent on environmental conditions that favour the rate of virus accumulation and spread, may result in systemic infection of cucumber plants.

## DISCUSSION

In this study we used immunocytochemical localization analyses and biological assays to examine the colonization of cucumber plants by CGMMV. The observed progress of CGMMV infection in cucumber plants is consistent with phloem transport from assimilate source to sink organs, as has been shown for TMV in *Nicotiana* species (Cheng *et al.*, 2000, and references therein). It has been well documented that virus phloem transport parallels photoassimilate transport (reviewed by Haywood *et al.*, 2002; Lucas & Wolf, 1999; Nelson & Van Bel, 1998; Oparka & Turgeon, 1999; Santa Cruz, 1999; Thompson & Schulz, 1999). Virus and assimilate phloem transport involves three steps: phloem loading in source organs, movement through the transport phloem and unloading in sink organs (Nelson & Van Bel, 1998; Oparka & Turgeon, 1999; Thompson & Schulz, 1999). In cucurbits, assimilate loading is presumably symplastic and the ICs of minor veins play a major role in the synthesis and loading of the transported oligosaccharides (Gross & Pharr, 1982; Haritatos *et al.*, 1996; Holthaus & Schmitz, 1991; Mitchell *et al.*, 1992; Turgeon, 1996). Similarly, it has been shown that CMV accumulates in the ICs of minor veins in inoculated cucumber cotyledons (Thompson & García-Arenal, 1998). We did not detect CGMMV accumulation in the ICs, suggesting that either CGMMV loading follows another route or its accumulation in the ICs is too transient to be traced by immunocytochemical methods. Our results suggest that the IC–SE complex of cucumber cotyledons acts as a symplastic domain that limits the accumulation of CGMMV, easily detected in the VP cells but not in the ICs (Fig. 2B). Thus, different cell complexes may be involved in the phloem loading of different viruses in cucumber with VP cells playing a role for CGMMV but not for CMV. Similarly, TMV has been reported to accumulate in the CCs of minor veins in inoculated leaves of tobacco (Ding *et al.*, 1996), while absence of virus accumulation in the CCs of inoculated leaves has been reported for the tobamovirus *Sunn-hemp mosaic virus*, the potyviruses *Potato virus Y* and *Peanut stripe virus* and the comovirus *Cowpea mosaic virus* in different host species of the Solanaceae and Fabaceae (Ding *et al.*, 1998; Silva *et al.*, 2002). Species in the Solanaceae and Fabaceae, at odds with those in the Cucurbitaceae, are apoplastic loaders of photoassimilates with type 2 veins, *sensu* Gamalei (Van Bel & Gamalei, 1992). The fact that CGMMV accumulation was detected in the ICs of minor veins in systemically infected mature leaves suggests an intriguing difference in function between cotyledon and leaf veins or between inoculated and systemically infected leaves. Our data indicate that in source cotyledons and leaves, phloem loading of CGMMV can also occur in the phloem of major veins (order III to IV), as reported for CMV and other viruses (Cheng *et al.*, 2000; Thompson & García-Arenal, 1998; Silva *et al.*, 2002) and as has been suggested to occur for sucrose loading in cucurbits (Turgeon, 1996).

Little is known about how viruses circulate in the transport

phloem and unload in systemically infected tissues (Oparka & Santa Cruz, 2000). CGMMV localized initially in the CCs of the external phloem and thereafter colonized the stem cortex, indicating that CGMMV unloads and probably reloads during its transport along the stem. Although graft experiments have shown that systemic movement in the internode does not require virus multiplication (Wisniewski *et al.*, 1990), a similar leaky pattern of transport has been described for photoassimilates (Nelson & Van Bel, 1998) and for TMV in tobacco (Susi *et al.*, 1999) or *Nicotiana benthamiana* (Cheng *et al.*, 2000). Virus multiplication in the CC and SE reloading could increase the efficiency of transport. It has been shown that CGMMV RNA circulates in the transport phloem in the form of virus particles (Simón-Buela & García-Arenal, 1999). Whether loading and reloading in the transport phloem involves uncoating and assembly of virions remains to be established. CGMMV localization in the external phloem contrasts with observations made for *Pepper mottle virus* or for TMV in the stem internal phloem of *Capsicum anuum* L. (Andrianifahanana *et al.*, 1997) or *N. benthamiana* L. (Cheng *et al.*, 2000), respectively. Virus and/or host factors determining differences in the stem transport route are yet to be identified but may depend on the plant vascular architecture and on the virus loading routes.

Symplastic unloading of *Potato virus X* in the major veins of *N. benthamiana* and of *Barley stripe mosaic virus* in barley longitudinal veins paralleled phloem unloading of carboxyfluoresceine (Haupt *et al.*, 2001; Roberts *et al.*, 1997). Symplastic unloading along major veins in source leaves has also been reported for other virus species and host plants (Cheng *et al.*, 2000; Ding *et al.*, 1998; Silva *et al.*, 2002). Our data also indicate that unloading of CGMMV in young developing leaves involves the major veins, where it was first detected. Our data showed two noticeable features. First, in systemically infected developing leaves, CGMMV was first detected in the abaxial phloem and in xylem cells (Fig. 1F). CGMMV accumulation was high in differentiating tracheids and immunogold-labelled particles were abundant in the lumen, the secondary cell wall and the middle lamella of intervascular pits (arrowheads in Fig. 2G), indicating a xylem tropism. Secondly, the temporal pattern of CGMMV immunodetection in different organs (Table 1) suggested that CGMMV could be unloaded in fully expanding, mature leaves, which may have undergone the sink to source transition. These two features suggest a function for the xylem in systemic movement of CGMMV, as reported for other viruses (Dubois *et al.*, 1994; Jones, 1975; Schneider & Worley, 1959a).

The involvement of phloem and xylem in the systemic movement was analysed using plants in which live cells in a portion of the 1st internode had been killed by steam treatment. Results indicated that CGMMV moved through the phloem of untreated controls, as was also the case for CMV, a virus previously reported to move exclusively through the phloem (Caldwell, 1930). However, systemic infection of upper leaves in steamed plants occurred at 29 °C, indicating xylem transport. Xylem transport in steamed plants was temperature dependent and inefficient, as deduced from the low percentage of steamed plants that became systemically infected (about 11 %) and from the delay in infection compared with untreated controls (Table 3). Long-distance movement through the xylem has been proposed for some viruses, but few reports have addressed this issue directly (Chambers & Francki, 1966; Dubois *et al.*, 1994; Jones, 1975; Opalka *et al.*, 1998; Schneider & Worley, 1959a, b; Verchot *et al.*, 2001). Xylem localization of virus particles has been reported for a number of viruses and hosts (e.g. Fribourg *et al.*, 1987; Khan *et al.*, 1994; Robertson & Carroll, 1989; Russo *et al.*, 1967; Urban *et al.*, 1989). Also, virus particles were recovered from guttation fluid in cucumber plants infected with 12 different viruses including CGMMV (French & Maureen, 1999). Since guttate originates from xylem exudate, this was considered evidence of xylem transport for these viruses. Interestingly, the presence or absence of *Brome mosaic virus* particles in guttation fluid of different hosts correlated with virus-induced damage of cells – mainly xylem cells – in the veins suggesting a mechanism for virus exit to the apoplast (Ding *et al.*, 2001). Nevertheless, xylem localization need not result in infection via the xylem. Indeed, our data show that CGMMV RNA was present in the xylem of steamed plants at 24 and 29 °C, but systemic infection via the xylem only occurred at 29 °C.

The mechanisms of xylem transport of plant viruses remain largely unexplored. It could be that entry into the xylem occurs in differentiating xylem elements. Thus, it has been proposed that infection of the root tips before xylem elements differentiate is required for systemic movement of *Beet necrotic yellow vein virus* through the xylem (Dubois *et al.*, 1994). This should not be a limiting factor in our system, as CGMMV was detected by Western blot and Northern hybridization (Fig. 4) in root tips. A common feature of viruses reported to translocate through the xylem is the high stability of the virus particles and their resistance to proteases (Gergerich, 2002), a feature shared by tobamoviral particles (Klug, 1999), which would be a necessary characteristic to withstand the action of proteases during programmed cell death during tracheary element development (Kozela & Regan, 2003). It is more difficult to envision how viruses would exit the xylem after transport and infect the protoplasts of living cells. A current hypothesis is that calcium binding by virus particles will disrupt pit membranes between mature and differentiating xylem vessels or tracheids, so enabling the virus to traffic through the membrane. This hypothesis was proposed on the basis of immunochemical analyses of the colonization of rice plants by *Rice yellow mottle virus* (Opalka *et al.*, 1998) and on the well-known role of calcium in the stabilization of *Sobemovirus* isometric capsids (Hsu *et al.*, 1976; Opalka *et al.*, 2000). It is known that calcium binding stabilizes TMV particles (Gallagher & Lauffer, 1983; Namba *et al.*, 1989), and putative calcium binding sites have

been described for CGMMV, watermelon strain (Wang & Stubbs, 1994), which has the same amino acid sequence in the CP as the SH strain (Ugaki *et al.*, 1991). We could speculate that the higher efficiency of xylem transport of CGMMV at 29 versus 24 °C could be related to a greater virus accumulation and, hence, higher calcium binding in the xylem and/or to relaxation of the pit membrane at the higher temperature.

Thus, our data show that systemic movement of CGMMV in cucumber plants occurs through the phloem and, less efficiently, through the xylem. How general this pattern of virus infection is remains to be explored.

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