The Bundle Sheath-Phloem Interface of *Cucumis sativus* Is a Boundary to Systemic Infection by Tomato Aspermy Virus

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Accepted 15 October 1997.

The progress of infection of two cucumoviruses in cucumber plants was analyzed immunohistochemically. Strain Fny of cucumber mosaic virus (CMV, FFF) was found to infect cucumber tissues systemically by 6 days postinoculation (dpi), while a reassortant virus with RNAs 1+2 of Fny-CMV plus RNA3 of strain 1 of tomato aspermy virus (FFT) was unable to move long distance and infect cucumber plants systemically. FFF infection of the vasculature was detected 6 dpi in the phloem of a low percentage of both minor (order VII–VI) and major (order V–IV) veins. At 9 dpi, infection was detected in phloem cells of about 50% of both minor and major veins. FFT colonization of inoculated cotyledons followed a pathway similar to that of FFF, but virus accumulation was never detected in vascular tissues. In minor or major veins, FFT infection was arrested at the bundle sheath (BS), and at 9 dpi was not detected in intermediary or other phloem cells. Thus, our data indicate that the BS-phloem interface is a boundary for the systemic movement of these viruses in cucumber, and provide evidence of a functional difference between the plasmodesmata connecting mesophyll cells, as well as mesophyll and BS cells, which allow the movement of both FFF and FFT, from the plasmodesmata connecting BS and phloem, which allow movement of FFF but not of FFT.

Additional keywords: long-distance movement, phloem loading, tomato aspermy cucumovirus.

The progress of the infection of plants by most viruses occurs through two main processes: (i) cell-to-cell movement through parenchymatic tissues, from the initially infected cells to the vasculature; and (ii) long-distance movement through the phloem leading to the systemic infection of the susceptible host (for a recent review see Carrington et al. 1996). During the last 10 years much information has been obtained on the role of virus-encoded movement proteins (MPs), and of host cell factors, in promoting the cell-to-cell spread of virus infection through the plasmodesmata. This information has been crucial to understanding not only the life cycle of plant viruses but also the role of plasmodesmata in intercellular communi-

cation in plants (Gilbertson and Lucas 1996; Lucas et al. 1996). Much less is known about systemic movement: this usually occurs via the phloem and it follows the path of photoassimilates (Leisner et al. 1992; Matthews 1991). Also, the analysis of virus/host systems in which systemic movement is impaired has brought evidence for the need of specific host and virus factors, different from MP, for systemic movement to occur (Carrington et al. 1996; Séron and Haenni 1996). Systemic movement is a key process in the virus life cycle, as plants in which viral infection is limited to the initially infected leaves are functionally resistant under natural conditions, i.e., show no symptoms and are poor sources for virus spread in plant populations. Also, knowledge of the factors allowing, or preventing, viral access to, and transport in, the phloem, may contribute to understanding phloem transport in general (Gilbertson and Lucas 1996; Stitt 1996).

Cucumber mosaic and tomato aspermy cucumoviruses (CMV and TAV, respectively) are two members of the well-characterized *Cucumovirus* genus of plant viruses (Palukaitis et al. 1992). In their messenger-sense, three-component RNA genome, movement-related functions are encoded in RNAs 2 and 3. Protein 2b, encoded by RNA2, is needed for long-distance movement in some host plants (Ding et al. 1995b). Protein 3a, encoded by RNA3, is the MP, required for cell-to-cell movement, with RNA-binding and plasmodesmata-gating properties (Kaplan et al. 1995; Li and Palukaitis 1996; Ding et al. 1995a). The coat protein (CP), also encoded by RNA3, is needed for both cell-to-cell and long-distance movement (Boccard and Baulcombe 1993; Suzuki et al. 1991). RNA1 may also have a role in the systemic accumulation of CMV (Gal-On et al. 1994). Work from our laboratory has shown that the CP is also a host-specific determinant for systemic movement: CMV and TAV share a range of host plants, and are both able to replicate and spread cell to cell within the inoculated cotyledons or inoculated leaves of cucumber (*Cucumis sativus* L.). In this host plant, though, only CMV infection is systemic, and CMV CP complements the systemic infection of TAV (Taliany and García-Arenal 1995). The infection of cucumber cotyledons with CMV or TAV is therefore an excellent model system for the analysis of systemic movement of viral infection.

Cucurbits are symplastic phloem loaders (Turgeon 1996), and vein anatomy of cucurbits has been described in detail (Schmitz et al. 1987; Turgeon et al. 1975). Briefly, minor

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veins (order VI and VII) are bounded by a single layer of bundle sheath (BS) cells, are bicollateral, and consist of xylem tracheary elements, phloem parenchyma (PP), sieve elements (SE), and companion cells (CC). CC on the abaxial phloem are larger than ordinary CC, have dense cytoplasm, and are connected by numerous plasmodesmata to both BS and SE. These cells are called intermediary cells (IC; *sensu* Turgeon et al. 1993) and are considered to play an important role in symplastic phloem loading of photoassimilates (Beebe and Turgeon 1992; Haritatos et al. 1996; Schmitz et al. 1987). In major veins of order V and IV, in which CC are still in contact with the BS, large IC and typical CC coexist in the abaxial phloem. In lower order veins (order I to III), not considered to be involved in photoassimilate loading (Schmitz et al. 1997), no IC are found.

We present here data on the progress of cucumber infection by CMV, and on the limitation of systemic infection associated with TAV CP. The BS/phloem interface is a boundary for viral systemic movement, as has also been reported for other plant/virus systems (Ding et al. 1995c, 1996; Goodrick et al. 1991; Wintermantel et al. 1997). All other reports, to our knowledge, involve host plant species that are apoplastic phloem loaders, with important differences in minor vein anatomy and function, while this is the first report in which limitation of systemic movement is analyzed in a symplastic loader.

RESULTS

Analysis of FFF and FFT infection in cucumber plants.

The progress of the infection in cucumber plants of two cucumoviruses was analyzed. Fny-CMV virion RNA derived from biologically active, full-length cDNA clones representing RNAs 1, 2, and 3 of strain Fny of CMV is called FFF, and virion RNA of the reassortant virus derived from biologically active clones representing RNAs 1 and 2 of Fny-CMV and RNA3 of strain 1 of TAV is called FFT (see Materials and Methods).

Young cucumber plants were inoculated by gently rubbing FFF or FFT RNA on the adaxial face of fully expanded cotyledons. At the time of inoculation the first true leaf was expanding and had a length of 1 to 1.5 cm at its main vein (order I). FFF-inoculated cotyledons developed chlorotic spots by 3 to 5 days postinoculation (dpi). Systemic symptoms were first observed in the first true leaf 6 to 8 dpi as a vein-clearing. Expansion of the second leaf started 4 to 5 dpi; this leaf first developed a vein-clearing and then a mosaic during 8 to 14 dpi. Vein-clearing and mosaic developed in all subsequently formed leaves. Cotyledons of plants inoculated with FFT RNA showed chlorotic spots, similar to those induced by FFF, at 4 to 7 dpi. No symptoms were observed in true leaves even 6 weeks after inoculation.

The presence of virus in the leaves of FFF- or FFT-inoculated plants was analyzed by immunodetection of their CPs in protein extracts (Fig. 1). The reactivity of each antiserum with the homologous CP (i.e., CMV or TAV) was assayed in Western blots (immunoblots) against a range (0.125 to 1 µg) of purified CP. The curves relating protein content of each electrophoretic band and its densitometric measurement of absorbance were not significantly different (not shown). Thus, both antisera showed the same reactivity with the homologous CP. Both CMV and TAV CP were clearly detected 3 dpi in the

inoculated cotyledons. For both FFF and FFT, CP accumulation in cotyledons grew to reach a maximum between 6 and 12 dpi and decreased afterwards. FFF CP was detected first in the second leaf at 6 dpi, and antigen accumulation increased until 12 dpi and decreased afterwards (Fig. 1A). FFT CP was not detected at the second true leaf during this period (Fig. 1B). These data indicate that FFT does not move systemically in cucumber plants.

Immunohistochemical analysis of FFF and FFT infection of cucumber cotyledons.

The accumulated CP of both FFF and FFT in infected cells was clearly detected with a light microscope with antibodies obtained against 1-TAV or Fny-CMV CPs (compare control in Figure 2A with other panels in Figure 2; note that the TAV CP antibody showed a nonspecific reaction with xylem, Fig. 2D–F). FFF infection was detected 3 dpi in the cotyledonary areas that started to develop in chlorotic spots. Virus infection was found in groups of epidermal and palisade mesophyll (PM) cells, and in spongy mesophyll (SM) underneath. In the SM, infection progressed toward the abaxial side of veins (not shown). Between 3 and 6 dpi, infection progressed in the mesophyll. At 6 dpi, infection had reached very few veins: of 26 veins observed from 17 different specimens only one major vein of order IV (60 to 80 vascular cells) and one minor vein of order VI (11 to 20 vascular cells) were found infected. In these veins, virus accumulation was detected in the BS cells, in the abaxial vascular parenchyma (VP) cells of the major vein, and in the PP and IC of the minor vein (Table 1). By 9 dpi, viral infection was present in most mesophyll cells of chlorotic areas, and in a high percentage of the veins. Virus antigen was detected in about 50% of minor veins of order VII (4 to 10 vascular cells) and VI, and in about 50% of major veins, of which 70% were of order V (30 to 55 cells). In minor veins, virus accumulation was found in about 60% of the BS and PP cells, and in more than 80% of IC (Table 1; Fig. 2B). In major veins, virus infection was detected in all types of cells in the abaxial phloem (Table 1; Fig. 2C). Thus, virus access to the vasculature and, presumably, to SE, at early times

Fig. 1. Progress of infection by FFF or FFT (see text for definitions) in cucumber. Protein extracts from 0.002 g of inoculated cotyledons (lanes 1, 3, 5, and 7), or from the second true leaf (lanes 2, 4, 6, and 8) of plants inoculated with FFF (A) or FFT (B) were probed with antibodies against Fny-CMV coat protein (CP) or 1-TAV CP, respectively. Proteins were extracted at 3 (lanes 1 and 2), 6 (lanes 3 and 4), 12 (lanes 5 and 6), or 20 (lanes 7 and 8) days postinoculation.

after inoculation occurred at both minor and major veins, probably depending on the random localization of the infection sites on the cotyledonary lamina. As infection progressed, a larger percentage of veins was infected, similarly for both minor and major veins. For both major and minor veins, virus infection always progressed from the abaxial BS and vascular cells, as indicated by Figure 2C.

Infection by FFT progressed slightly more slowly than by FFF, but followed the same path: from the epidermis to underlying PM and SM, and to the abaxial side of veins (Fig. 2D). In contrast to FFF, no antigen was found in vascular cells. Viral infection progressed to BS cells, but not to inner vascular cells. In minor veins, viral antigen was never detected in the IC or PP cells, even when mesophyll and BS cells were heavily infected by 9 dpi (Fig. 2E). In major veins, virus infection did not progress farther than the abaxial BS (compare Figure 2F with 2C). Immunoelectron microscopy confirmed that no virus antigen was found in IC, even when present in BS cells (Fig. 3). IC were easily identified in the electron microscope, even in major veins, by their dense cytoplasm with small vacuoles (not shown) and by the abundant plasmodesmata in the cell wall abutting to adjacent BS cells that branch out on the IC side (Fig. 3). Note that by 9 dpi the proportion of major veins in which virus was detected at the BS was similar for FFF and FFT (Table 1). At 9 dpi, the proportion of infected BS cells in minor veins, but not in major veins, was also similar for both viruses. Thus, data indicate that the lack of systemic movement of FFT is not due to the inability to reach the vasculature, but that the BS cells limit further virus movement.

DISCUSSION

Systemic or long-distance spread of virus infection in host plants is a poorly understood process. Its analysis is essential to understanding plant virus relationships, and may provide insights into the mechanisms of supracellular control over long-distance transport of photoassimilates (Lucas et al. 1996). The analysis of the progress of CMV infection in cucumber plants shows that systemic movement from the initially inoculated cotyledons into the true leaves had occurred by 6 dpi. At this time, virus accumulation was detected in the phloem of a small fraction of both minor (order VII–VI) and major (order V–IV) veins. Virus movement in the phloem appears to mostly parallel the movement of photoassimilates (Leisner et al. 1992; Oparka et al. 1995), and in *Cucumis* spp. it has been proposed that veins of order VII to IV are involved in photoassimilate loading (Schmitz et al. 1987). The observed pathway of virus infection from the initially infected adaxial epidermis and PM to the abaxial side of veins has also been reported for other plant/virus systems (Goodrick et al. 1991; Wang et al. 1996), in which minor veins are collateral (i.e., lack adaxial phloem). It should be pointed out that in the bi-collateral veins of *Cucumis* spp. the abaxial phloem, but not the adaxial phloem, is involved in photoassimilate loading (Schmitz et al. 1987).

In contrast to infection by FFF, FFT infection in cucumber does not become systemic. Work in our laboratory had shown that 1-TAV was unable to systemically infect cucumbers, and that systemic infection was complemented by CMV CP (Taliany and García-Arenal 1995). In the present work, we

have used not 1-TAV but the pseudorecombinant virus FFT, since a role for CMV RNAs 1 and 2 in systemic movement has been reported (Ding et al. 1995b; Gal-On et al. 1994). As was the case for 1-TAV, FFT does not infect cucumbers systemically, although infection of the inoculated cotyledons was nearly as efficient as for FFF. The results we report show that FFT infection is stopped, or delayed, at the BS. However, our data do not exclude the possibility of FFT infecting phloem cells later than 9 dpi. They show, though, that the BS-phloem interface is a boundary for the systemic infection of cucumber by FFT: at 9 dpi, when FFF infection had already gone systemic for several days, and affected a large percentage of phloem cells in the veins of the inoculated cotyledons, FFT was not found in the inner vascular cells, although FFT infection was detected in the BS of a high percentage of veins. With one possible exception (Schaad and Carrington 1996), the BS-phloem interface was also found to be a barrier for virus movement in other reported analyses of viruses with impaired systemic movement. This was the case for various strains and mutants of tobacco mosaic tobamovirus (TMV) defective for systemic movement in tobacco, which infected VP and CC less efficiently than the wild-type TMV, while infecting BS cells with similar efficiencies (Ding et al. 1995c, 1996). Our results are also similar to those reported for cowpea chlorotic mottle bromovirus in soybean lines resistant or susceptible to systemic infection by this virus, in which resistance was associated with virus infection being stopped at the BS-vascular cell interface (Goodrick et al. 1991). The interface between BS and vascular cells has also been reported as the site of virus movement restriction in transgenic tobacco plants in which systemic movement of CMV was impaired (Wintermantel et al. 1997). Cowpea and tobacco, though, are apoplastic phloem loaders with type 2 veins (*sensu* Gamalei), in which symplastic connection between BS and the CC-SE complex is poor (Van Bel et al. 1992; Van Bel and Gamalei 1992). This, at least in part, may be related to the inhibition of virus trafficking at this interface (Wintermantel et al. 1997). Cucurbits are symplastic loaders, with type 1 veins, and abundant plasmodesmata occur at the BS/IC interface (Turgeon et al. 1975; Turgeon and Hepler 1989; Turgeon et al. 1993). There is evidence for differences in the trafficking ability of the plasmodesmata connecting mesophyll cells and BS, BS and IC, or the various phloem cell types, in cucurbits and other symplastic loaders (Beebe and Turgeon 1992; Kempers et al. 1993; Haritatos et al. 1996). Differences in plasmodesmata permeability delimiting symplastic domains is needed for symplastic phloem loading (Turgeon 1996). Our data also support functional differences between the plasmodesmata connecting mesophyll and BS cells, which allow movement of both FFF and FFT, and those connecting BS and phloem cells, which allow movement of FFF but not of FFT. The CP has been shown to be a host-specific determinant for systemic movement in the analyzed system (Taliany and García-Arenal 1995), but the role of CMV CP in promoting trafficking at the BS/phloem interface remains to be determined.

MATERIALS AND METHODS

Viruses and inoculations.

Recombinant plasmids with cDNA clones representing the full-length RNAs 1, 2, and 3 of Fny-CMV (pF109, pF209, and

Fig. 2. Immunohistochemical analysis of infection by FFF or FFT (see text for definitions) in cucumber cotyledons. Samples were taken at 9 days postinoculation and immunostained with antibodies against Fny-CMV coat protein (CP) (A, B, C) or 1-TAV CP (D, E, F). A, Mock-inoculated control. B, Minor vein from FFF-inoculated cotyledon. C, Major vein from FFF-inoculated cotyledon. D, Section of FFT-inoculated cotyledon. E, Minor vein from FFT-inoculated cotyledon. F, Major vein from FFT-inoculated cotyledon. Bundle sheath cells (B), intermediary cells (I), and xylem tracheary elements (T) of minor veins are indicated in B and E. Some bundle sheath cells (B) and xylem tracheary elements (T) are also indicated in A, C, and F. Abaxial bundle sheath cell of major vein where FFT infection is arrested is indicated by an arrow in F. Bars indicate 15 μ m in D; 7.5 μ m in A, C, and F; and 5 μ m in B and E.

Table 1. Percentage of cells infected for different vein types in cucumber cotyledons inoculated with FFF or FFT RNA^a

Inoculum	dpi ^c	No. analyzed veins (samples)	Minor veins ^b				Major veins ^b		
			N ^d	BS ^e	PP ^e	IC ^e	N ^d	BS ^e	VP ^e
FFF	6	26 (17)	1/11 (9)	83	28	80	1/15 (6)	32	50
	9	35 (23)	8/17 (47)	64 $\pm$ 9	60 $\pm$ 16	83 $\pm$ 17	8/18 (44)	43 $\pm$ 7.5	49 $\pm$ 7
FFT	6	25 (14)	0/12 (0)	0	0	0	0/13 (0)	0	0
	9	36 (24)	4/15 (27)	73 $\pm$ 4	0	0	9/21 (43)	26 $\pm$ 3	0

^a See text for definitions of FFF and FFT.

^b Minor veins are veins of order VII and VI; major veins are veins of order V and IV (see text).

^c Days postinoculation.

^d N = number of veins in which infection had reached bundle sheath cells over total observed veins. In parentheses, same data expressed as percentages.

^e BS = percentage of infected bundle sheath cells; PP = percentage of infected phloem parenchyma cells; IC = percentage of infected intermediary cells; VP = percentage of infected abaxial phloem cells (vascular parenchyma, phloem parenchyma, and intermediary cells were pooled together since cell types were not unequivocally identified in all observed veins). These percentage values were computed for each vein in which infection had reached bundle sheath cells. For instance, in FFF-infected cotyledons at 6 dpi, only one major vein of order V was found infected; this vein was bound by 25 BS cells, and it had 44 VP+PP+IC. FFF CP was detected in 8 BS cells (32%) and in 22 VP+PP+IC (50%). When more than one vein was infected, mean $\pm$ standard errors were then computed

pF309) were the gift of Peter Palukaitis (SCRI, Dundee, UK) and have been described (Rizzo and Palukaitis 1990). Plasmid p13, representing full-length RNA3 of 1-TAV, has also been described (Moreno et al. 1997). RNA transcripts obtained from these plasmids in the presence of m7GpppG (New England Biolabs, Beverly, MA) were used to inoculate young tobacco (*Nicotiana tabacum* cv. Xanthi-nc) plants. Inocula consisted of mixtures of 5 µg of each transcript in 0.1 M Na₂HPO₄, in the combinations RNAs 1+2+3 of Fny-CMV (FFF) or RNAs 1+2 of Fny-CMV+RNA3 of 1-TAV (FFT). From the infected tobacco plants virions were purified according to Lot et al. (1972) for FFF, or Habili and Francki (1974) for FFT. RNA was extracted from purified virions with phenol-sodium dodecyl sulfate and used as inoculum for experiments with cucumber plants. Cucumber (*Cucumis sativus* cv. Ashley) plants were inoculated with 5 µl of virion RNA at 200 µg/ml in 0.1 M Na₂HPO₄ on the adaxial surface of fully expanded cotyledons approximately 10 days after sowing. Healthy or inoculated tobacco or cucumber plants were grown in a growth chamber at 25°C with a 14 h light / 10 h dark cycle.

Antisera and immunoblots.

Antisera to 1-TAV CP or to Fny-CMV virion particles were obtained in rabbits as described (Moreno et al. 1997). These sera were used for analysis in immunoblots of protein extracts from cucumber cotyledons or leaves, as described (Moreno et al. 1997), or for light and electron microscopy.

Fig. 3. Immuno-electronmicroscopic detection of FFT (see text for definition) infection in minor veins. Samples were taken at 9 days postinoculation from FFT-inoculated (A) or mock-inoculated (B) cotyledons. Parts of bundle sheath (BS) and intermediary (IC) cells are presented delimited by a cell wall area showing a deltoid plasmodesmata field. Bar represents 200 nm.

Microscopy.

Specimens with approximate dimensions of 0.5 × 2 mm were cut from cotyledons, fixed by vacuum infiltration in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.2, and left overnight in fresh fixative at 4°C. The specimens were then washed 3 times in the buffer alone, dehydrated in a series of methanol:water mixtures at room temperature, and embedded in LR White Resin (London Resin, Reading, UK) by incubating in a 50:50 mix of methanol:LR White for 2 h, then in pure LR White for 2 h, both steps at room temperature, and then overnight at 4°C in fresh LR White. Polymerization was carried out by incubating the specimens in sealed gelatin capsules for 24 h at 52°C.

For light microscopy, 1-µm-thick sections of embedded specimens were cut and stuck onto poly-L-lysine (Sigma, St. Louis, MO) coated slides. Sections were blocked for 1 h in Tris-buffered saline (TBS) pH 7.5, 0.1% Tween 20, 1% wt/vol bovine serum albumin (BSA) at room temperature, and then incubated with the primary antibody in the same buffer overnight at 4°C. The slides were then washed 5 × 10 min in TBS pH 7.5, 0.1% Tween, and then incubated with anti-rabbit gold conjugate (5 nm, Sigma) in blocking buffer for 1.5 h at room temperature. The slides were then washed 5 × 10 min in distilled water and silver enhanced (Biocell, Cardiff, UK) for approximately 10 to 15 min.

For electron microscopy, 100-nm-thick sections were cut and supported by Formvar-coated nickel grids. The blocking and labeling procedure was as for the light microscope, except that 10 nm gold was used. After gold labeling, grids were washed 3 × 5 min in distilled water, stained with a saturated uranyl acetate solution for 5 min, washed 3 × 1 min in distilled water, and dried.

All sections were cut with glass knives with a Reichert Ultramicrotome. Light microscopy was done with a Zeiss Axio-phot, and electron microscopy with a Jeol model JEM-1200EX II.

ACKNOWLEDGMENTS

This work was in part supported by grant BIO96-1026-C02-01, CICYT, Spain. J. T. was supported by HCM Contract CHR-X-CT94-0611, of the European Commission. We thank Peter Palukaitis, SCRI, Dundee, UK, for cDNA clones of Fny-CMV RNAs 1, 2, and 3, and Emilio Rodríguez-Cerezo, CNB, Madrid, for access to electron microscopy facilities.

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