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Tobamovirus and Potyvirus Accumulation in Minor Veins of Inoculated Leaves from Representatives of the Solanaceae and Fabaceae[1]

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Virus invasion of minor veins in inoculated leaves of a host is the likely prelude to systemic movement of the pathogen and to subsequent yield reduction and quality loss. In this study we have analyzed the cell number and arrangement in minor veins within mature leaves of various members of the Solanaceae and Fabaceae families. We then monitored the accumulation pattern of several tobamoviruses and potyviruses in these veins at the time of rapid, phloem-mediated movement of viruses. Vascular parenchyma cells were the predominant and sometimes only cells to become visibly infected among the cells surrounding the sieve elements in minor veins containing 9 to 12 cells. In no instance did we observe a companion cell infected without a vascular parenchyma cell also being infected in the same vein. This suggests that the viruses used in this study first enter the vascular parenchyma cells and then the companion cells during invasion. The lack of detectable infection of smooth-walled companion or transfer cells, respectively, from inoculated leaves of bean (*Phaseolus vulgaris*) and pea (*Pisum sativum*) during a period of known rapid, phloem-mediated movement suggests that some viruses may be able to circumvent these cells in establishing phloem-mediated infection. The cause of the barrier to virus accumulation in the companion or transfer cells, the relationship of this barrier to previously identified barriers for virus or photoassimilate transport, and the relevance of these findings to photoassimilate transport models are discussed.

Invasion of veins in an inoculated leaf is a prerequisite for the rapid dissemination of plant viruses throughout the host. Although systemic spread is an essential and major contributor to the decrease in crop yield and quality, it has not been extensively studied (for review, see Carrington et al., 1996; Gilbertson and Lucas, 1996; Séron and Haenni, 1996; Nelson and van Bel, 1998). Most plant viruses are transported only through the phloem of the host (Bennett, 1956; Hull, 1991). The accumulation of plant viruses in vein cells has been observed (Esau and Cronshaw, 1967; Esau et al., 1967; Kluge, 1967; Esau, 1968), but the emphasis of these studies was on the subcellular structures induced by the infection and not on the ontogeny of the infection within the veins of inoculated leaves during the period of rapid, phloem-mediated spread. In addition, the veins studied were usually not classified into particular types (i.e. classes or orders), nor was the relationship of vein types to their function in photoassimilate transport discussed (for review, see Hickey, 1979; Grusak et al., 1996). Only recently has research been published indicating that the minor veins in leaves progressing from net photosynthate importers to exporters (i.e. from sink to source tissue) are modified irreversibly to function as conduits for photoassimilate export (Turgeon, 1986; Schmalstig and Geiger, 1987).

Minor veins account for the majority of veins in a leaf (Horner et al., 1994; Grusak et al., 1996; and refs. therein) and therefore are the most likely to be affected by a spreading virus. Minor veins of herbaceous dicot plants are surrounded by BS cells, which enclose VP cells, C cells, SEs, and TEs. The number of cells in the minor veins, their structure, and the structure of the C cells are highly variable in different plant species (van Bel, 1993; Grusak et al., 1996).

C cells have been categorized into three major groups based on their structure and the number of plasmodesmal connections with cells other than SEs (Turgeon et al., 1975; Gamalei, 1989; Wimmers and Turgeon, 1991; for review, see Grusak et al., 1996; Nelson and van Bel, 1998, and refs. therein). Where PD connections are high, the C cells are called intermediary cells. Intermediary cells are found in representatives of the Cucurbitaceae and contain vesicular labyrinths that may be ER. C cells with 10- to 100-fold fewer plasmodesmal connections than observed for intermediary cells and containing smooth cell walls are referred to as smooth-walled C cells.

Smooth-walled C cells are observed in many plants from the Solanaceae (e.g. tobacco, *Nicotiana tabacum*, and tomato, *Lycopersicon esculentum*) and in some representatives from the Fabaceae (e.g. bean, *Phaseolus vulgaris*). C cells from the third category have almost no plasmodesmal connections with BS or VP cells (approximately 10-fold fewer connections than are observed for smooth-walled C cells) and are characterized by cell wall invaginations that vary in vol-

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Abbreviations: BS, bundle-sheath; C, companion; CP, coat protein; dpi, days postinoculation; PD, plasmodesmata; PStV, peanut stripe virus; PVY, potato virus Y; SE, sieve element; SHMV, sunnhemp mosaic virus; T, transfer; TE, tracheary element; TMV, tobacco mosaic virus; VP, vascular parenchyma.

ume with the rate of photosynthate flow. These C cells are referred to as T cells and are found in representatives from the Fabaceae (e.g. pea, *Pisum sativum*) and other families. The vast majority of plants containing smooth-walled C cells or T cells are thought to transfer carbohydrates into the SE/C cell complex apoplastically. The route that phloem-transported viruses take to invade minor veins of plants with apoplastic phloem loading of photosynthates is of interest because, to our knowledge, these viruses have only been shown to move through the symplasm.

We have previously focused much of our research on the accumulation of TMV in minor veins of tobacco (Ding et al., 1995, 1996b). In this species the ratio of VP to C cells infected in inoculated leaves ranged from approximately 3:1 to 8:1 after inoculation with wild-type virus. In minor veins from inoculated leaves of potato, more VP cells than C cells were infected by an unknown virus, but the results were not quantitated or shown (McCauley and Evert, 1989). Carrington and co-workers have studied the accumulation of tobacco etch potyvirus in minor veins of *N. tabacum* (Cronin et al., 1995; Schaad and Carrington, 1996). They did not quantitate their results using large sample numbers or differentiate between VP and C cells, but the virus appeared to be in both VP and C cells of the infected plants.

Because of the accessibility of minor veins to virus invasion, the ability of TMV to accumulate in a strikingly greater number of VP cells compared with C cells in *N. tabacum*, and the potential impact of virus transport on photoassimilate transport, we initiated a survey of the accumulation pattern by various tobamoviruses and potyviruses in minor veins of hosts differing in their minor vein structures and C cell morphologies. Conclusions are drawn about the accessibility of certain cell types to virus infection, the path of virus movement in minor veins, and the potential impact of this movement on photoassimilate transport.

MATERIALS AND METHODS

Viruses and Plants

TMV (U1 strain) and SHMV were obtained in purified forms as described previously (Nelson et al., 1993; Deom et al., 1994). PVY and the "blotch" isolate of PStV were obtained from Dr. John Sherwood (Oklahoma State University, Stillwater) as infected leaf tissue and maintained in *Nicotiana benthamiana*. Purified TMV or SHMV or crude extracts from *N. benthamiana* leaves infected with PVY or PStV were used as virus inocula. Crude extracts were prepared by grinding systemically infected leaf tissues in 5 volumes of 0.05 M sodium phosphate buffer, pH 7.0, per gram fresh weight of tissue.

Six species of plants from two different families (Solanaceae and Fabaceae) were studied: pepper (*Capsicum annuum* L. cv Marengo), tomato (*Lycopersicon esculentum* L. cv Rutgers), tobacco (*Nicotiana tabacum* L. cv Xanthi nn; hereafter referred to as Xanthi), *N. benthamiana*, bean (*Phaseolus vulgaris* L. cv Tendergreen), and pea (*Pisum sativum* L. cv Rondo).

C. annuum, L. esculentum, N. benthamiana, and Xanthi plants were grown as described for Xanthi (Ding et al., 1995). For *P. vulgaris* and *P. sativum*, seeds were germinated on moist tissue paper in plastic containers and then transplanted at 2 d after germination into 6.5-inch pots (four plants per pot) containing an artificial soil medium (Metro-Mix 350, Grace, Marysville, OH). Growth conditions for *P. vulgaris* and *P. sativum* were the same as for the solanaceous plants.

Plant Inoculation

For experiments with TMV, PVY, or PStV, four or five plants were inoculated with a virus and the experiment was repeated once. In each experiment two plants were mock inoculated (0.05 M sodium phosphate buffer, pH 7.0) for use as negative controls. For TMV, the fully expanded fourth and fifth leaves of 5-week-old *N. benthamiana* and Xanthi plants, and the first and second fully expanded true leaves of 4-week-old *C. annuum* and *L. esculentum* plants were dusted with Carborundum (320 grit, Fisher Scientific) and then mechanically inoculated with purified virus (25 μg of virus/mL; 30 μL of inoculum/leaf). The inoculated leaves were immediately rinsed with distilled water and the plants were placed in a growth chamber (16/8 h of light/dark at 24°C). PPFD was 152 μmol photons m^{-2} s^{-1}. For PVY and PStV, *N. benthamiana* or Xanthi plants were mechanically inoculated (50 μL/leaf) and grown as described for experiments with TMV.

For experiments with SHMV, *P. vulgaris*, *P. sativum*, and *N. benthamiana* plants were inoculated. For *P. vulgaris* and *P. sativum*, 10 plants with similar physiological development, determined by estimating plastochron indices (Erickson and Michelini, 1957), were chosen at 15 d after planting. Each group of 10 plants was then divided into two equal-sized subgroups with one subgroup being inoculated 1 d before the other. Fully expanded primary leaves of *P. vulgaris* and the first and second trifoliolate leaves of *P. sativum* were inoculated with purified SHMV (25 μg/mL, 30 μL/leaf). Leaves of *N. benthamiana* were inoculated with SHMV (25 μg/mL, 30 μL/leaf) as described for TMV. Growth conditions for plants infected with SHMV were similar to those for plants infected with TMV.

Antisera and Biotinylated-RNA Probe

Polyclonal antisera against SHMV and TMV were produced as described previously (Deom et al., 1994; Ding et al., 1995). Antisera against PVY and PStV were obtained from the American Type Culture Collection (PV AS-50a, Rockville, MD) and Dr. Brandt Cassidy (Samuel Roberts Noble Foundation, Inc., Ardmore, OK), respectively. The PStV antiserum was produced by Drs. R. Naidu and D.V.R. Reddy (International Crops Research Institute for the Semi-Arid Tropics, Andhra Pradesh, India). All antisera were used without further purification.

A biotinylated RNA probe, complementary to nucleotides 1 to 3332 of the TMV genome, was prepared from a cDNA clone of the masked strain of TMV using an in vitro transcription kit (MAXIscript, Ambion, Austin, TX) and

biotin-14-CTP (Life Technologies) as described previously (Ding et al., 1996a).

Tissue Sampling, Fixation, and Embedding

Inoculated leaves were sampled and assessed for virus accumulation at the times designated for each experiment. Systemically infected leaves of *P. vulgaris* and *P. sativum* were also sampled and analyzed. Sampled leaf tissue, cut into 3- × 6-mm segments, was fixed either with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0, for 10 s using a microwave oven as described previously (Ding et al., 1995), or with 3% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 4 h at 4°C. Tissues from TMV-inoculated *C. annuum*, *L. esculentum*, and *N. benthamiana* leaves were fixed using the microwave technique. After three washes (10 min each) in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2.5% Suc, the tissues were dehydrated in a series of ethanol solutions, infiltrated, and embedded in LR White resin (London Resin Co. Ltd., London, UK) as described previously (Ding et al., 1995).

Immunocytochemistry

Tissues prepared from *N. benthamiana*, *C. annuum*, and *L. esculentum* leaves inoculated with TMV were analyzed by immunocytochemistry and light microscopy as described previously (Ding et al., 1995), except that the dilutions for the first and second antibodies were 1:5,000 and 1:200, respectively. Tissues prepared from *N. benthamiana* leaves inoculated with SHMV and *N. benthamiana* and Xanthi leaves inoculated with PVY were analyzed by double-sided labeling immunocytochemistry and light microscopy (Ding et al., 1996a). Antisera against SHMV or PVY were used at dilutions of 1:500 and 1:800, respectively. Tissues from *P. vulgaris* and *P. sativum* leaves inoculated with SHMV and *N. benthamiana* leaves inoculated with PStV were analyzed by immunocytochemistry and electron microscopy as described previously (Ding et al., 1996b). Antisera against SHMV or PStV were used at dilutions of 1:2,000 and 1:10,000, respectively.

The size and cellular composition of the veins in mature leaves from different hosts were determined by examining sections under a light microscope. Cell types of minor veins in *N. benthamiana*, *C. annuum*, and *L. esculentum* leaf tissues were identified by plasmolysis using 1 M sorbitol treatment (Ding et al., 1995). Cell types of minor veins in *P. vulgaris* and *P. sativum* leaf tissues were identified based on the appearance of the cells under the electron microscope and in comparison with previously reported results (Gamalei, 1989).

In Situ Hybridization

Sections (2 μm) from Xanthi leaf tissues inoculated with TMV or buffer (i.e. mock inoculated) were analyzed for viral RNA accumulation using a biotinylated RNA probe complementary to the masked-strain TMV genome, followed by incubation with a streptavidin-gold (5 nm) conjugate (BioCell Research Laboratories, Cardiff, UK), and then silver enhancement as previously described (Ding et al., 1996a).

RESULTS

Symptom Ontogeny

The type of symptom induced by the viruses on inoculated host plants and their time of appearance were determined (Table I). The viruses either induced chlorotic lesions not later than 4 dpi or induced no symptoms throughout the observation period (approximately 10 dpi) on the inoculated leaves. On young, systemic (uninoculated) leaves, all viruses induced visible symptoms of varying types by 6 dpi.

Survey of Vein Size and Architecture in Different Hosts

Minor veins are generally accepted to be the sites for phloem loading of photoassimilates and constitute 90 to 95% of the total leaf vein length in dicotyledons (Grusak et al., 1996). As such, they would be the first veins that a virus would come into contact with during cell-to-cell spread, and would thus represent a potential point for virus entry into the vascular system. To provide a reproducible basis for comparisons of virus infection of minor veins between our studies and those of others, we conducted an abbreviated survey of the cell numbers and cell arrangements within the smaller veins of the six species in this study.

We reported previously that approximately 90% of minor veins in sections prepared from mature Xanthi leaves had 9 to 12 cells (i.e. class V vein, as defined by Ding et al. [1988, 1995]). Typical class V veins in mature *N. tabacum* leaves consist of a file of xylem TEs and two SEs, with the SEs surrounded by a single layer of alternating VP and C cells (3 of each) (Ding et al., 1995, 1996b). In the current

Table I. *Symptom ontogeny in leaves from various hosts after virus infection*

Virus and Host	Leaf	
	Inoculated	Young systemic
TMV		
N. benthamiana	c.l.[a] 3 dpi	v.c.[b] 5 dpi
N. tabacum	c.l. 3 dpi	v.c. 5 dpi
C. annuum	c.l. 3 dpi	v.c. 5 dpi
L. esculentum	n.s.[c]	v.c. and l.r.[d] 5 dpi
SHMV		
N. benthamiana	n.s.	v.c. 5 dpi
P. vulgaris	c.l. 4 dpi	m.m.[e] 6 dpi
P. sativum	n.s.	v.c. 6 dpi
PVY		
N. tabacum	n.s.	v.c. 5 dpi
N. benthamiana	n.s.	m.m. 5 dpi
PStV		
N. benthamiana	n.s.	s.c.[f] 5 dpi

[a] c.l., Chlorotic lesion. [b] v.c., Vein clearing. [c] n.s., No symptoms. [d] l.r., Leaf rolling. [e] m.m., Mild mosaic. [f] s.c., Systemic chlorosis.

work we have examined sections prepared from leaf tissues of the six plant species by light microscopy. For convenience we have categorized the smaller veins from these species into four groups by cell number only: category VI, consisting of fewer than 8 cells; category V, 9 to 12 cells; category IV, 13 to 18 cells; and category III, more than 19 cells. For these species, these categories of veins generally represent minor veins (veins embedded in the mesophyll cells). This classification does not take into account the lengths of veins or their branching patterns. Also, although our vein category numeration system (e.g. category V) is equivalent to the vein class numeration system defined for *N. tabacum* (Ding et al., 1988; e.g. class V), it is not equivalent to the vein classification system described for *P. sativum* (Wimmers and Turgeon, 1991; e.g. categories VI, V, and IV ≅ class IV).

Xanthi and *N. benthamiana* minor veins were mostly category V (approximately 88% of 338 veins and approximately 69% of 134 veins, respectively). Electron microscopic examination of six category V veins from five sections prepared from sorbitol-treated leaf tissues of *N. benthamiana* indicated that cells at positions 4, 6, and 8 were plasmolyzed, but not cells at positions 5, 7, and 9 (Fig. 1, A and B). Therefore, by previously described criteria (Turgeon and Hepler, 1989; Beebe and Evert, 1992; Ding et al., 1995), cells at positions 4, 6, and 8 were determined to be VP cells and cells at positions 5, 7, and 9 were determined to be C cells. The C cells contained smooth walls (Fig. 1, A and B). Further examination of the category V veins from *N. benthamiana* showed that approximately 91% of these veins had the same cellular arrangement as typical class V veins observed in Xanthi leaves (Ding et al., 1995).

In contrast to the *Nicotiana* species, only 36% of the 90 veins observed in sections from *C. annuum* were category V. Within the category V veins, 66% had the same cellular arrangement observed for typical Xanthi class V veins (e.g.

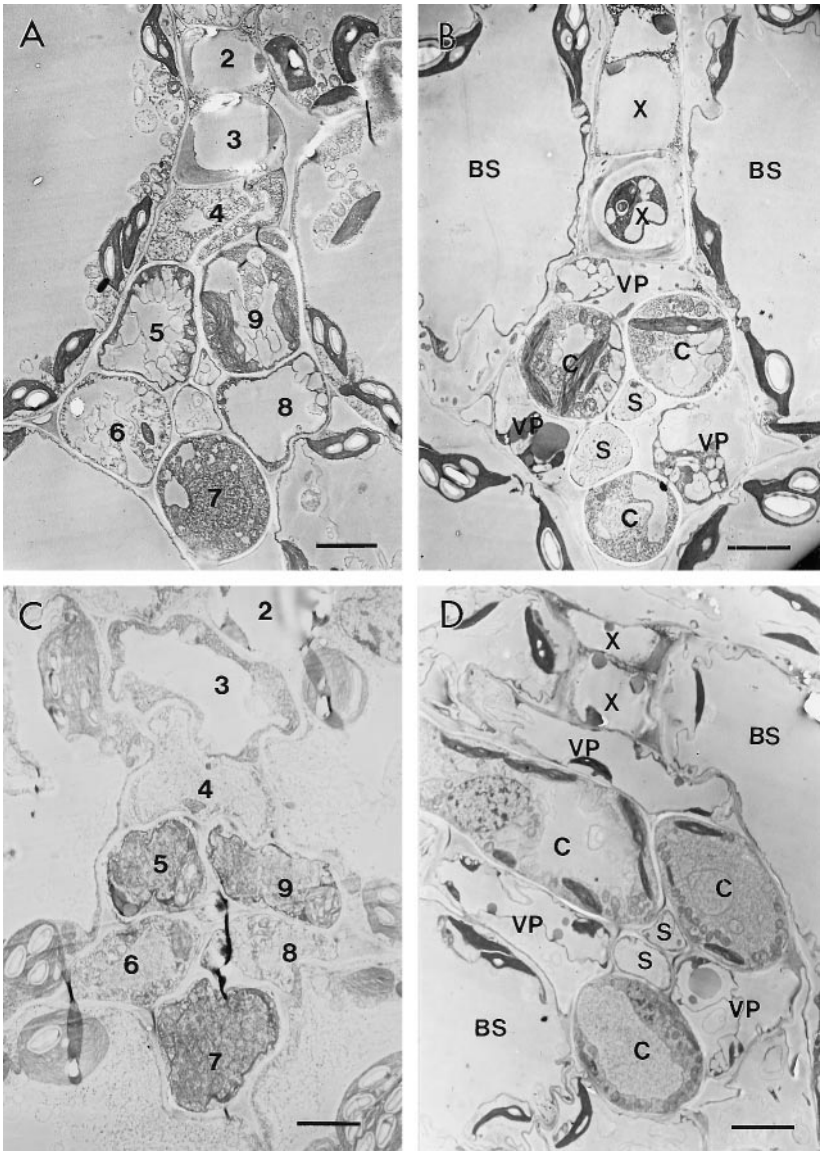

Figure 1. Electron micrographs of typical category V veins (9–12 vein cells, not including BS cells) in sections from mature leaves of *N. benthamiana* (A and B) or *C. annuum* (C and D) treated with distilled water or 1.0 M sorbitol solution before fixation. A and C, Veins treated with distilled water before fixation. Cells are numbered to allow text discussion. Note in A that there are two cells at the position of cell 4. B and D, Veins treated with 1.0 M sorbitol before fixation. Note that the plasmalemma of cells 4, 6, and 8 are separated from their cell walls and thus plasmolyzed, whereas those of cells 5, 7, and 9 are not. Therefore, cells 4, 6, and 8 are considered to be VP cells, and cells 5, 7, and 9 are considered to be C cells. S, SE; X, xylem TE. A and B, bars = 3 μm; C and D, bars = 4 μm.

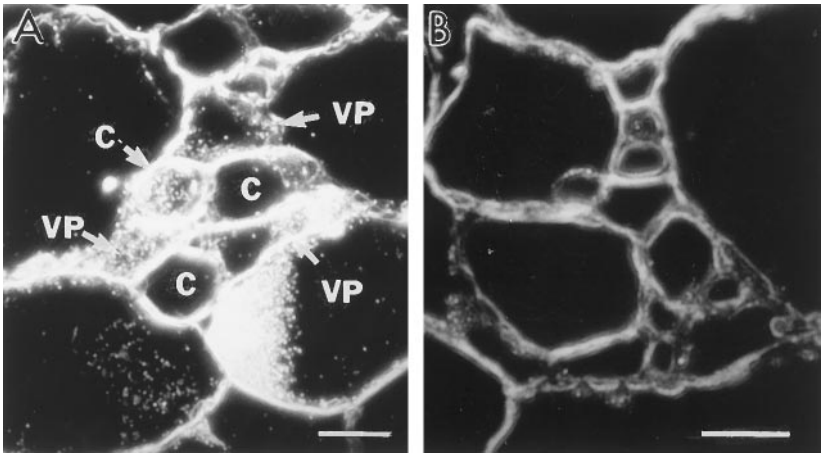

Figure 2. Accumulation of genomic TMV RNA in cells of a category V vein (defined in Fig. 1 legend) from a mature inoculated leaf of Xanthi at 4 dpi. A, Section from an inoculated leaf analyzed by in situ hybridization using a biotinylated-RNA probe complementary to nucleotides 1 to 3332 of the TMV genome. The biotinylated-RNA probe was then detected using a streptavidin-gold conjugate followed by silver enhancement. The section was photographed under a light microscope. The labeling signal appears as white specks in cells in the dark-field image. Three VP cells and one C cell of the vein had accumulated viral RNA. The infected C cell is denoted with an arrow. B, Section from a mock-inoculated Xanthi leaf analyzed as for the vein in A. TMV RNA was not detected in vein cells from this section. Bars = 12 μm.

Fig. 1, C and D). The other 34% of the veins were atypical, containing either an extra cell within the ring of VP and C cells or a xylem TE at position 4. C cells from *C. annuum* were also smooth-walled (Fig. 1, C and D).

L. esculentum contained a similar percentage of category V veins as *C. annuum* (approximately 39% of 61 veins). Of these veins, approximately 33% had cellular arrangements identical to those of class V veins of Xanthi except that cells at position 4 were often xylem TEs (as opposed to the VP cells observed for the *Nicotiana* species; data not shown). The C cells contained smooth walls as in the other Solanaceae species examined.

In the legumes approximately 56% of the 93 veins observed in sections from *P. vulgaris* were category V, whereas 14% of the 69 veins observed in sections from *P. sativum* were category V. The cell composition and/or vein structure in leaves of *P. vulgaris* and *P. sativum* were not similar to those examined in the Solanaceae. For both species, cell types were not regularly organized within the veins, thus eliminating the possibility of cell identification through light microscopy and positional analysis, as could be accomplished for the Solanaceae. Results from electron microscopic analyses showed that veins in *P. vulgaris* leaves had VP cells, SE/smooth-walled C cell complexes, and xylem TEs, as reported previously (Gamalei, 1989). Veins in *P. sativum* had VP cells, SE/T cell complexes, and xylem TEs, as was also reported previously (Wimmers and Turgeon, 1991). The presence of cell wall ingrowths in cells in *P. sativum* veins is diagnostic of T cells.

In our survey of virus invasion of minor veins, we focused most of our observations on the category V veins because they represent minor veins for these species and we had identified the cell types and their positions within these veins either by light microscopy or electron microscopy.

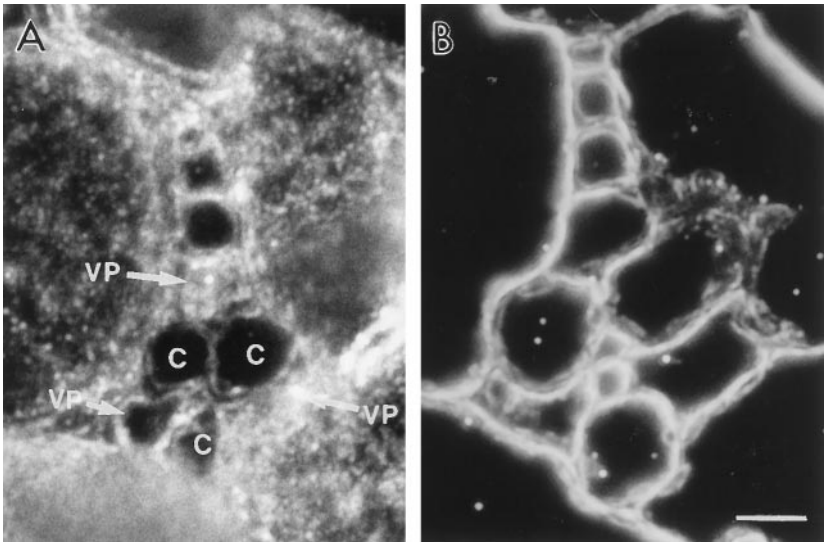

Figure 3. Accumulation of TMV in cells of a typical category V vein (defined in Fig. 1) from a mature inoculated leaf of *C. annuum* at 3 dpi. TMV accumulation was visualized by immunocytochemistry with primary antibody against TMV and secondary antibody conjugated with gold followed by silver enhancement. The sections were photographed under a light microscope. The labeling signal appears as white specks in cells in the dark-field image. A, Section from TMV-inoculated leaf; B, section from a mock-inoculated *C. annuum* leaf. Bar = 7.1 μm.

Table II. *Analysis of minor veins for virus accumulation in VP cells, C/T cells, or both*

Plant	dpi	Virus	No. of Veins Examined	Veins with Only VP Cells Infected	Veins with Only C or T Cells Infected	Veins with Both VP and C or T Cells Infected
				%		
N. benthamiana	3	TMV	43 (10)[a]	62.8	0	37.2
C. annuum	3		37 (21)	45.9	0	54.1
L. esculentum	3		4 (8)	75.0	0	25.0
N. benthamiana	4	SHMV	40 (9)	62.5	0	7.5
P. vulgaris	5		22 (8)	31.1	0	0
P. sativum	5		19 (8)	94.7	0	0
Xanthi	4	PVY	18 (5)	38.8	0	61.2
N. benthamiana	4		17 (10)	41.2	0	58.8
N. benthamiana	4	PStV	23 (5)	30.4	0	56.5

[a] Numbers in parentheses indicate the number of sections analyzed.

In Situ Hybridization

We determined in our previous work that early after infection, the TMV CP and the 126-/183-kD proteins accumulate in more VP cells than in C cells of class V veins (Ding et al., 1995). To provide further evidence that VP cells are more efficiently infected by TMV than C cells during the process of minor vein invasion, we analyzed 27 veins from 10 chlorotic lesions induced on TMV-inoculated Xanthi leaves at 4 dpi using a biotinylated RNA probe complementary to the virus genome. Of the veins that accumulated viral RNA, the ratio between infected VP and C cells was nearly 5:1. Approximately 37% of the typical (i.e. containing the correct cell number and arrangement) category V veins examined contained TMV genomic RNA in VP cells, but not in C cells; 15% of the veins contained viral RNA in both VP and C cells, and none of the veins contained viral RNA only in C cells (for an example of in situ hybridization, see Fig. 2). This result corresponds to our previous results for TMV CP accumulation (Ding et al., 1995) and suggests that VP cells are infected early after inoculation, preferentially and before C cells. No viral RNA was detected in sections prepared from mock-inoculated tissues (Fig. 2B).

Immunocytochemistry

When sections prepared from *N. benthamiana*, *C. annuum*, and *L. esculentum* leaves inoculated with TMV were examined under the light microscope for TMV CP accumulation, strong labeling (i.e. white specks in cells attributable to silver enhancement of the gold conjugated to the second antibody) was seen in the cell interior of infected mesophyll and vein cells with dark-field illumination (Fig. 3 and data not shown). No signal was seen in cells of sections prepared from mock-inoculated *N. benthamiana*, *C. annuum*, and *L. esculentum* leaves (Fig. 3B and data not shown). In *N. benthamiana* leaves infected with TMV, 63% of the typical category V veins contained TMV CP in the VP cells but not in the C cells (Table II). No category V veins in these sections accumulated TMV CP in C cells alone. The ratio of infected VP versus C cells was 11:1 (Table III). In *C. annuum* leaves, 46% of the typical category V veins were found to contain TMV CP in VP cells but not in C cells (Table II; Fig. 3), and, as was observed for *N. benthamiana*, no veins contained virus in C cells alone. The ratio of infected VP versus C cells was approximately 3:1 (Table III). For *L. esculentum*, only four typical category V veins were observed. Of these, three contained TMV CP in only VP cells, and none con-

Table III. *Percentage of infected VP and C/T cells within minor veins and their ratio*

Plant	dpi	Virus	No. of Veins Examined	Cell Type		VP:C/T[b]
				VP	C/T[a]	
				%		
N. benthamiana	3	TMV	43	73.7	7.0	11:1
C. annuum	3		37	78.2	28.8	3:1
L. esculentum	3		4	100.0	8.3	12:1
N. benthamiana	4	SHMV	40	61.5	2.5	25:1
P. vulgaris	5		22	31.1	0.0	>31:1
P. sativum	5		19	90.3	0.0	>90:1
Xanthi	4	PVY	18	37.0	9.3	4:1
N. benthamiana	4		17	34.0	5.9	6:1
N. benthamiana	4	PStV	23	58.7	29.6	2:1

[a] All species analyzed had smooth-walled C cells except *P. sativum*, which had T cells. [b] Ratio of VP to C/T cells infected.

tained TMV in only C cells (Table II). The ratio of infected VP to C cells was approximately 12:1 (Table III).

Minor veins within sections from mature leaves of *N. benthamiana*, *P. vulgaris*, and *P. sativum* inoculated with the tobamovirus SHMV were analyzed for CP accumulation. Because SHMV caused no visible symptoms in inoculated leaves of *N. benthamiana* and *P. sativum*, leaf tissues from these two hosts were sampled randomly the day before the systemic leaves showed vein clearing or mild mosaic symptoms, a stage at which vascular movement of virus is imminent or has occurred. In *N. benthamiana*, about two-thirds of the veins contained SHMV CP in VP cells but not in C cells (Table II; Fig. 4). No vein contained virus in C cells only. The ratio between infected VP and C cells in the typical category V veins examined was approximately 25:1 (Table III). A total of 22 veins (2 category VI veins, 14 category V veins, and 6 category IV veins) from inoculated leaves of *P. vulgaris* and 19 veins (1 category VI vein, 9 category V veins, 5 category IV veins, and 4 category III veins) from leaves of *P. sativum* were analyzed by immunogold labeling and electron microscopy. Within these veins the virus CP was detected only in VP cells and not in smooth-walled C cells (*P. vulgaris* leaves; Table II; Fig. 5) or T cells (*P. sativum* leaves; Table II; Fig. 6A). No immunogold labeling signal was seen in cells of healthy control sections (data not shown).

To determine if SHMV was capable of accumulating in any smooth-walled C cells or T cells, we examined seven minor veins from leaves of *P. vulgaris* and five from *P. sativum*, all infected by vascular movement. Approximately 25% of C cells (*P. vulgaris* leaves; data not shown) and 47% of T cells (*P. sativum* leaves; see Fig. 6B as an example) examined contained the virus. Therefore, the virus is capable of accumulating in these cells in uninoculated leaves.

Potyvirus accumulation in plants from the Solanaceae was also studied. In Xanthi and *N. benthamiana* leaves inoculated with PVY, approximately 39 and 41%, respectively, of the category V veins contained PVY CP in VP cells only (Table II; Fig. 7). No veins examined contained PVY CP in only C cells for either species (Table II). Ratios between the infected VP and C cells in category V veins of the sections from the two plants were approximately 4:1 and 6:1, respectively (Table III). Experiments with *N. benthamiana* leaf tissue inoculated with PStV resulted in a greater number of minor vein cells infected and a ratio of infected VP to C cells of approximately 2:1 (Table III).

DISCUSSION

In this study we determined that plant viruses representing the Sindbis virus (i.e. TMV and SHMV) and Picornavirus (i.e. PVY and PStV) supergroups were predominantly detected in VP cells relative to smooth-walled C cells in category V minor veins from mature inoculated leaves of a common host (e.g. *N. benthamiana*; Tables II and III). TMV or PVY were also detected predominantly in VP cells versus smooth-walled C cells of *C. annuum*, *L. esculentum*, and Xanthi (Tables II and III). In *P. vulgaris* and *P. sativum* infected with SHMV, virus was detected exclusively in VP cells (Table II). An inability of the virus to replicate in C cells seems an unlikely explanation for the results because we did observe the virus in a low number of smooth-walled C cells within inoculated leaves of Solanaceae plants (Tables II and III) and in approximately 25 and 47% of the smooth-walled C and T cells from systemically infected leaves of *P. vulgaris* and *P. sativum*, respectively (Fig. 6B and "Results"). Therefore, unless a subtle developmental change occurs within C cells in mature leaves over time to control virus replication, the results indicate that a barrier exists for virus entry into mature smooth-walled C cells or T cells. This barrier to virus accumulation in C or T cells was functional when systemic movement of the viruses through phloem was occurring. It operates at a different location from a previously identified cellular interface that regulates virus and photosynthate transport, the BS cell/internal vein cell interface (Goodrick et al., 1991; Ding et al., 1992, 1995; Sanger et al., 1994; Russin et al., 1996; Wintermantel et al., 1997; for review, see Nelson and van Bel, 1998).

Whether the barrier to virus accumulation in the SE/C cell complex represents a passive defense by the host based

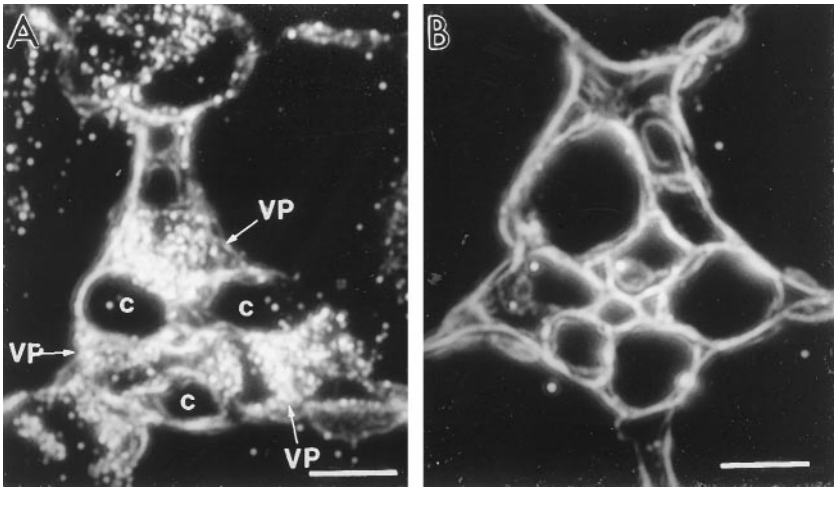

Figure 4. Accumulation of SHMV in a typical category V vein (defined in Fig. 1) from a mature inoculated leaf of *N. benthamiana* at 4 dpi. SHMV accumulation was visualized as described in Figure 3 using antibody against SHMV. A, Section from leaf tissue inoculated with SHMV. Virus was detected in three VP cells but not in C cells of this vein. B, Section from a mock-inoculated leaf tissue analyzed as for the vein in A. Bars = 12 μm.

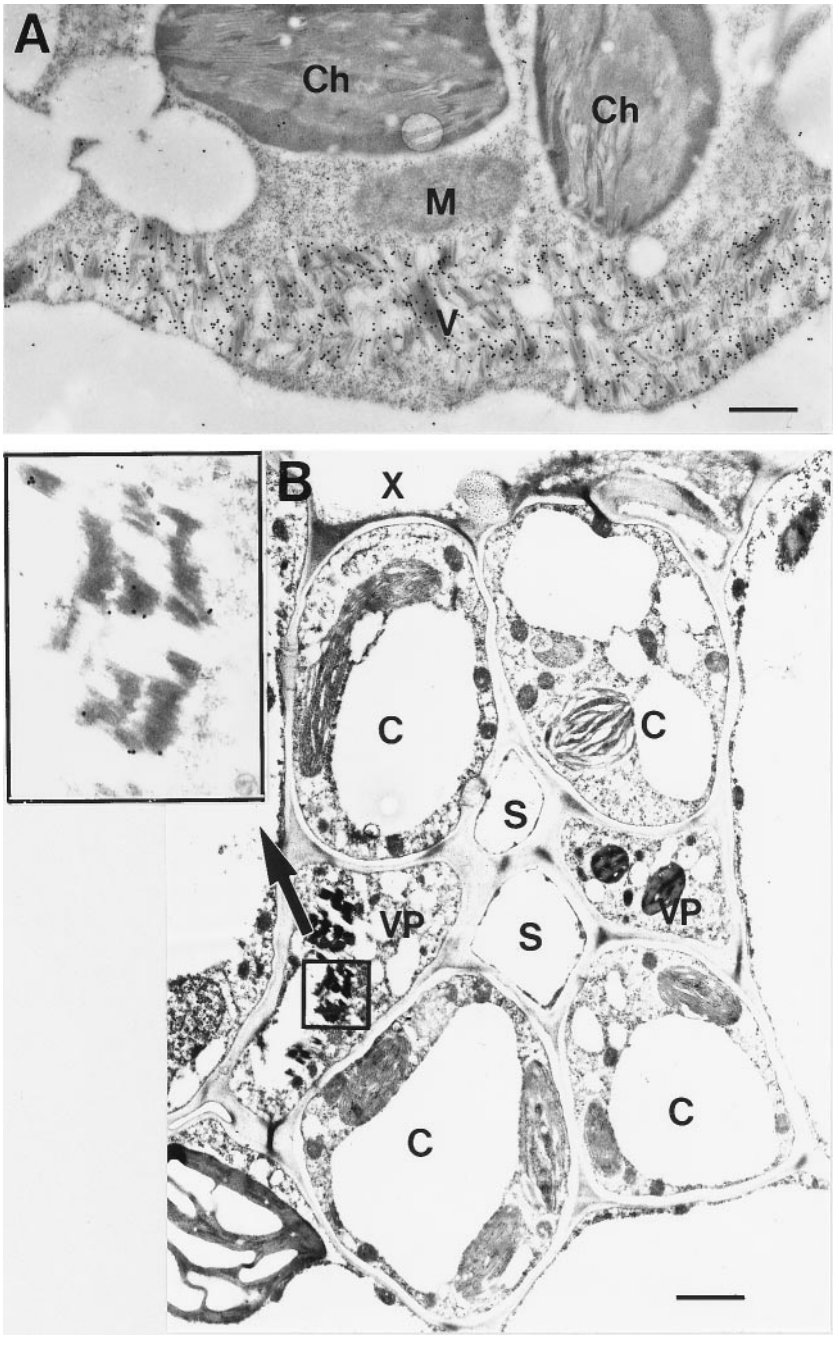

Figure 5. Accumulation of SHMV in cells from a *P. vulgaris* leaf at 5 dpi. Accumulation of SHMV in cells was analyzed by immunocytochemistry using an antibody against SHMV and a goat anti-rabbit gold (20 nm) conjugate. Sections were examined and photographed under an electron microscope. A, Virion (V) aggregates labeled with immunogold in the cytoplasm of an infected mesophyll cell. Ch, Chloroplast; M, mitochondrion. Bar = 0.7 μm. B, Virion aggregates labeled with immunogold in the cytoplasm of a VP cell from a category V vein. No virion aggregates were detected in C cells of the vein. Inset, Magnification of several aggregates labeled with immunogold. S, SE; X, xylem TE. Bar = 2 μm.

on the limited number or altered structure of PD between these cells and the other vein cells or an active defense mounted in this region by these cells remains to be determined. Certainly the greater number of C versus T cells infected with virus positively correlates with the known increase in the number of PD between C cells versus T cells and other cells for the vein configurations tested (van Bel and Gamalei, 1992). However, the lack of any virus in T cells of *P. sativum*, which has been reported to contain the same number of PD between the SE/T cell complex and other cells compared with the SE/C cell complex and other cells of *N. tabacum* (van Bel and Gamalei, 1992), may indicate that PD numbers alone do not fully account for the lack of infection.

The structure of PD between C or T cells and other cells has been studied for only a few species. For *N. tabacum* and *P. sativum*, the PD between C or T cells and VP or BS cells are generally unbranched and not noted as being different from PD connecting VP and BS cells, or VP or BS cells themselves (Ding et al., 1988; Wimmers and Turgeon, 1991). However, in more detailed studies, structural differences were observed for PD between C cells and VP or BS cells compared with those at other cellular interfaces in *Moricandia arvensis* (Beebe and Evert, 1992). The PD be-

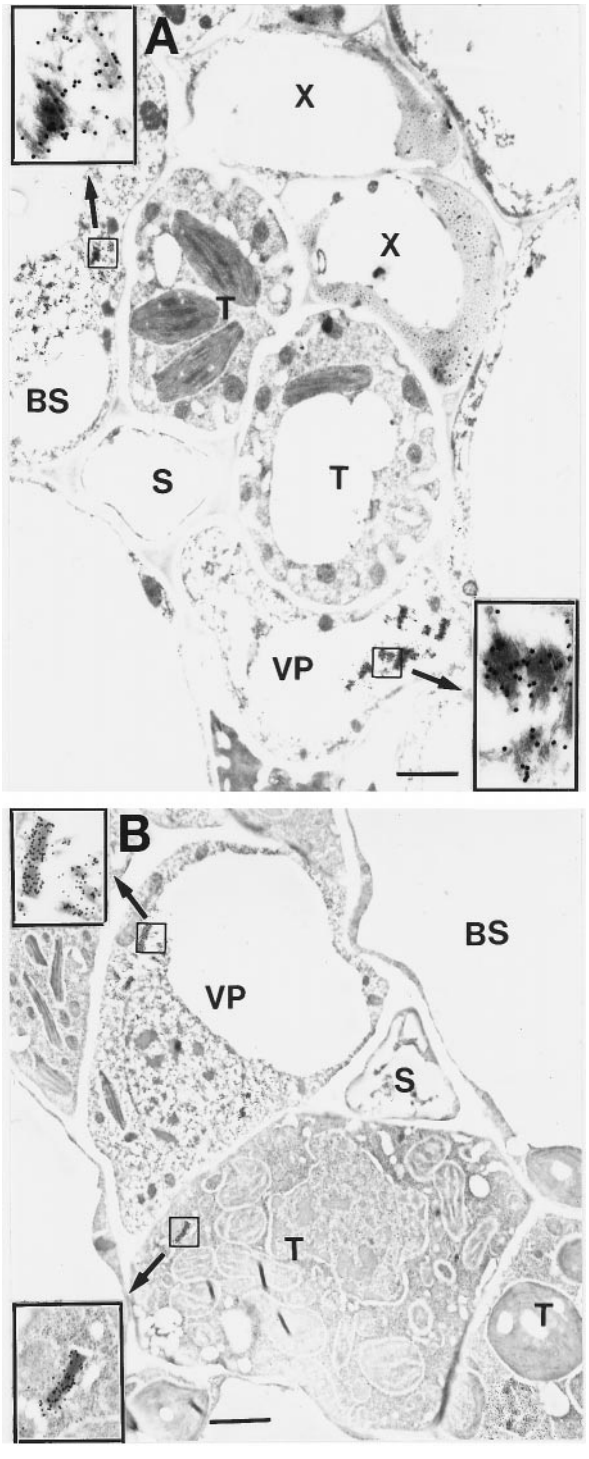

Figure 6. Accumulation of SHMV in minor vein cells of *P. sativum*. Accumulation of SHMV in cells was analyzed as described in Figure 6. A, Accumulation of SHMV in a category V vein from a mature inoculated leaf at 5 dpi. SHMV was detected in a BS cell, a VP cell, but not in the adjacent T cells. Bar = 1.5 μm. B, Accumulation of SHMV in a category VI vein from a systemically infected leaf at 8 dpi. Viral aggregates of SHMV were detected in both VP and T cells of the vein. Bar = 2 μm. Arrows point to magnified images of boxed areas showing immunogold labeling. S, SE; X, xylem TE.

tween C cells and VP or BS cells in *M. arvensis* lacked observable desmotubules and appeared to be occluded on the C cell side of the wall. Considering the need to maintain differing osmotic potentials between the SE/C cell complex and the other vein cells in plants that apoplastically load photosynthates, it is not surprising that the PD between these are modified. Further work is necessary to determine if such structural features exist for the PD between the C cells and other vein cells in the species we studied and whether such structures could impede transport of virus into the C or T cells of these species.

Recently, Callaway et al. (1996) determined that lack of movement by cauliflower mosaic virus in a resistant Arabidopsis ecotype correlated with greater gene expression or protein accumulation of general defense-response genes (e.g. pathogenesis-related proteins). It has also been determined that a specific host-resistance gene to a fungal pathogen encodes a protein, PRms, which localizes to PD in maize radicles (Murillo et al., 1997). If a host-resistance response to virus infection specific to the PD between C cells and VP or BS cells is present, it would be unusual in that it functions in different plant families and against viruses with different structures and replication and translation strategies. It would also be developmentally controlled, because some C cells become infected in (a) inoculated leaves of some species and (b) systemically infected leaves of species having no infection in the C cells in the inoculated leaves (Fig. 6; Table II; Ding et al., 1995). Further work is necessary to decipher whether the results obtained in this study reflect passive or active resistance.

In addition to identifying an intercellular barrier defining two cellular domains for virus accumulation, our findings with *N. tabacum* and *N. benthamiana* infected with TMV and SHMV, respectively, suggest that these viruses accumulate in VP cells before C cells (Table II and "Results"). However, the limited number of PD between C and BS cells could have limited the number of C cells infected relative to the VP cells, which are well connected with BS cells. This would decrease the chance of observing the infection of C cells before VP cells. In the legume species, the very limited number of plasmodesmal connections between C or T cells and cells other than the SEs may have limited the infection rate in these cells to a level that we were unable to observe in the population sampled. Further work is necessary to verify whether VP cells are infected before C cells in the dicotyledons.

Regardless of the order in which vascular cells become infected, the rate of systemic movement of SHMV in the legume species, in which C or T cells were not infected, is similar to that observed in the solanaceous host *N. benthamiana*, in which C cells were infected. This indicates that rapid phloem-dependent movement likely occurs in plants in the absence of C or T cell infection. Routes for vascular invasion by viruses without C cell infection include entrance to SEs at the termini of minor veins, directly through VP cells, or at branch points in veins where gaps may exist between VP and C cells, allowing mesophyll or BS cells to abut SEs. Esau (1967, 1977) observed that the vein endings of minor veins in *Beta vulgaris* and *Syringa* leaves have mesophyll cells abutting SEs. In *P. vulgaris* and

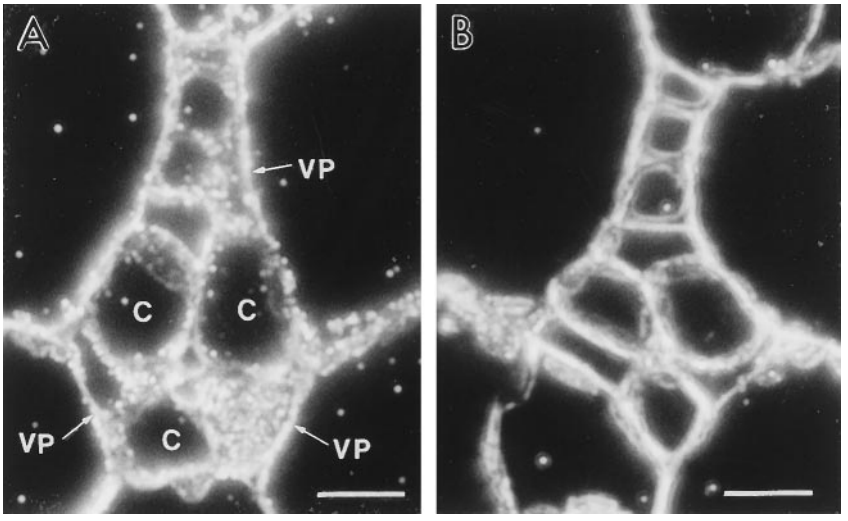

Figure 7. Accumulation of PVY in cells of a typical category V vein from a mature inoculated leaf of Xanthi at 4 dpi. Analysis for PVY accumulation was done as described in Figure 3 using an antibody against PVY. A, Section from leaf tissue inoculated with PVY. The virus was detected in three VP cells but not in C cells of this vein. B, Section from a mock-inoculated leaf analyzed identically to tissue shown in A. Bars = 10 μm.

C. annuum SEs are sometimes found in the vein endings (de Morretes, 1962). Therefore, in species having such a vein structure, the virus would not have to pass through C or VP cells to gain access to SEs.

Some plant species, however, have very few SEs that extend to the termini of the minor veins. A few plant species have 10% or fewer minor veins with termini, and less than 3% of these have SEs extending to the vein termini (Horner et al., 1994). In species containing veins with few termini, the minor veins that encircle the areole likely serve as the loading point for carbohydrate and virus transport. In these species viruses would need to gain access to the SEs through VP or C cells, because these cells generally encircle the SEs (see Figs. 1–7; also see Esau, 1967; van Bel, 1993). PD connections exist between VP cells and SEs in minor veins of various species, including *P. sativum* (Fisher and Evert, 1982; Evert and Mierzwa, 1986; Wimmers and Turgeon, 1991; Beebe and Evert, 1992), although in some species they have not been observed (e.g. *Solanum tuberosum* and *Vicia faba*; Gunning et al., 1974; McCauley and Evert, 1989; Grusak et al., 1996). In species with connections between VP cells and SEs, virus would not have to pass through C cells to gain access to SEs for phloem-dependent infection of uninoculated tissue. Virus-induced dilation of PD between VP cells and SEs would allow for the entry of virus progeny into the SEs.

Virus entry into SEs may occur at branch points in veins, however, the literature is sparse and mixed on the occurrence and functional significance of such gaps (for review, see Nelson and van Bel, 1998).

In systemically infected leaves, C cells of minor veins may become infected by various routes. Ding et al. (1995) found that the proportion of infected VP cells to infected C cells is approximately 1:1 in class V minor veins of *N. tabacum* leaves systemically infected with TMV. In this report we observed a higher proportion of smooth-walled C cells and T cells infected in systemically infected leaves compared with inoculated leaves of *P. vulgaris* and *P. sativum*. Our initial interpretation of these results was that virus could infect C cells by moving out of the SEs of minor veins through the abundant PD between these cells. Support for this route of virus infection comes from several studies on various plant species indicating that the molecular exclusion limits of PD at the SE/C cell interface are of a molecular mass greater than 3 kD and less than 40 kD (Kempers et al., 1993; Kempers and van Bel, 1997), as opposed to the approximately 0.8 kD observed for PD between mesophyll cells. Also, proteins in the range of 35 kD have been shown to move between these two cell types in *Triticum aestivum* (Fisher et al., 1992). Because of the large molecular exclusion limits between these cells it was reasonable to assume that the virus may establish a systemic infection through this route.

However, Roberts et al. (1997) have recently determined that potato virus X exits from large veins (predominantly order III and larger) exclusively in sink leaves of *N. benthamiana*. Thus, in this scenario, virus moves from major veins through mesophyll cells and infects cells within minor veins by passage through BS cells. Access to C cells would be gained through the more extensive network of PD believed to exist between all cells in immature leaves (for review, see Turgeon, 1989). Further research is necessary to finalize any conclusion in this area.

Aside from indicating potential transport routes for viruses through the vasculature, this study provides information relevant to researchers studying carbohydrate transport routes through this tissue. For example, the structure of T cells in *P. sativum*, with their limited symplastic connections to BS and VP cells, has been taken as one of several indicators that sugars pass through an intercellular space to enter the SE/T cell complex in this species (i.e. they are apoplastic SE loaders as opposed to symplastic loaders; Wimmers and Turgeon, 1991). Because systemic viral infections occur in similarly rapid time frames for species with widely varying numbers of plasmodesmal connections between the C or T cell complex and VP or BS cells, it appears that viruses have either used or introduced an efficient symplastic pathway for their movement. A symplastic pathway for macromolecular movement could affect an apoplastic photosynthate transport pathway by

allowing transient leakage of carbohydrates and other metabolites and ions into and out of the SE/T cell or SE/smooth-walled C cell complex. Even if leakage of photosynthate or host factors does not occur, transport of charged virions into SEs would affect the chemical gradient between the SE/C or T cell complex and the external vein cells. It will be important to determine the site and mechanism of virus transport into the SE/C cell complex and the effect of this transport on the osmotic and electrical gradients within phloem cells of the host.

In summary, our results indicate that both tobamoviruses and potyviruses accumulate in VP cells preferentially and possibly before infecting C cells in minor veins of members of the Solanaceae and Fabaceae families. The presence of a boundary between two cellular domains—the mesophyll, BS, VP cell domain, and the SE/smooth-walled C cell or/T cell complex domain—for virus accumulation is apparent for both virus types and coincides with previously suggested symplastic domains for photoassimilate transport. Our results suggest that for some plant species there may be transport of viruses from VP cells directly to the SE, but this requires further study. In addition, these results may affect current dogma on photoassimilate transport.

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