Spatial and Temporal Distribution of Bean Dwarf Mosaic Geminivirus in *Phaseolus vulgaris* and *Nicotiana benthamiana*

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ABSTRACT

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The distribution of the bipartite geminivirus bean dwarf mosaic (BDMV) within tissue and cell types of systemically infected *Phaseolus vulgaris* and *Nicotiana benthamiana* plants was determined at 6, 12, and 21 days post sap-inoculation (dpi) using an immunolocalization technique with a capsid protein antibody. In both hosts, BDMV was detected in most cell types of the inoculated leaves. In systemically infected immature and mature leaves of *P. vulgaris* (6 and 12 dpi) and in immature leaves of *N. benthamiana* (12 dpi), BDMV was detected in epidermal, mesophyll, bundle sheath, phloem parenchyma, and companion cells. However, by 21 dpi, BDMV appeared to have become restricted to phloem parenchyma, companion, and bundle sheath cells in newly developing leaves of infected *P. vulgaris* plants. Two distinct BDMV infection domains were identified in both hosts: a phloem domain that included protophloem and mature phloem cells, and a nonphloem domain that included bundle sheath and mesophyll cells and, to a lesser extent, epidermal cells. BDMV showed a striking tropism for the protophloem cells of the shoot apex, and the overall number of infected phloem cells decreased sharply from the apex and upper stem to the roots. The results of this study establish that BDMV infection is not phloem-limited in either host, which is consistent with the efficient sap-transmissibility of BDMV, and suggest that the developmental stage of the host can influence the cell/tissue distribution of the virus. A model for the establishment of systemic BDMV infection in sap-inoculated plants is presented.

Additional keywords: phloem-limitation, viral capsid protein, virus movement.

After a virus has been introduced into a plant, it must carry out a series of functions to achieve a systemic infection including replication, cell-to-cell movement, and long-distance transport (3,23). In general, plant viruses appear to be replication-competent in protoplasts prepared from a range of cell types including nonhost plants. Thus, the extent of infection of a virus in a susceptible host plant must be dependent upon the specific cell types through which it can move. It is now established that interactions between viral and plant host factors play an important role in determining whether a virus can move within a specific plant species (3). Viral movement proteins (MPs) play a central role in cell-to-cell movement (2,5,14,23,24). In many cases, this process involves the interaction of MPs with plasmodesmata, an increase in plasmodesmal molecular size exclusion limit (SEL), and the cell-to-cell trafficking of MPs and nucleic acids (7,8,28). However, plasmodesmata that interconnect different cell types are not identical and may be responsible, at least in part, for the establishment of specialized cellular and tissue domains within the plant, across which plant viruses may or may not be able to move. For example, within a leaf, the following tissue domains can be identified: mesophyll cells (MC)/bundle sheath cells (BS), BS/phloem parenchyma (PP), and companion cell (CC)/sieve element (SE). These domains have been explored in studies using plant viruses, as well as with transgenic plants expressing viral MPs (6,20,21,25,34). However, for most plant viruses, a complete understanding of the systemic infection process has yet to be developed.

We seek to develop an understanding of how plants are systemically infected by bean dwarf mosaic geminivirus (BDMV). Geminiviruses are a unique group of plant viruses characterized by their single-stranded DNA genome and small, twinned icosahedral virions (11,36,37). In nature, geminiviruses are transmitted by whiteflies or leafhoppers and cause economically important diseases of many crop plants. Most whitefly-transmitted geminiviruses have a bipartite genome and infect dicots, whereas leafhopper-transmitted geminiviruses are monopartite and most commonly infect monocots. It is generally accepted that infections by whitefly-transmitted geminiviruses are phloem-limited (specific examples: bean golden mosaic virus [19], Euphorbia mosaic virus in *Euphorbia heterophylla* [16], mungbean yellow mosaic [10], Jatropha mosaic virus [15], Indian cassava mosaic virus [30], and Abutilon mosaic virus [13]) (10,11,36,37). Although there are reports that infections by some whitefly-transmitted geminiviruses are not phloem-limited, these reports were based on infections of experimental host plants; i.e., Euphorbia mosaic virus in *Datura stromonium* (17) and tomato golden mosaic geminivirus in *Nicotiana benthamiana* (27,33). In a comparative study with tomato yellow mosaic geminivirus, infection was phloem-limited in the natural host tomato, but not in the experimental host, *N. glutinosa* (22).

BDMV provides a useful system in which to examine the cellular and molecular basis of the geminivirus-plant interaction, because it is well characterized on the molecular level and readily sap-transmitted to *Phaseolus vulgaris*, a natural host (defined here

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as a host that may be infected with a virus in nature), and to *N. benthamiana*, an experimental host (defined here as a host known to be infected with a virus under laboratory conditions) (9,26). BDMV represents a typical bipartite geminivirus, having a DNA-A component that encodes for replication and encapsidation functions and a DNA-B component that encodes two proteins (BR1 [BV1] and BL1 [BC1]) that coordinate viral nuclear and plasmodesmal transport, respectively (12,28). In the present paper, we report on the spatial and temporal analysis of BDMV infection in sap-inoculated *P. vulgaris* and *N. benthamiana* plants and show that BDMV is not phloem-limited in either host. Furthermore, the infection process in both hosts appears to involve groups of cells and cell types (domains) within phloem and nonphloem tissues.

MATERIALS AND METHODS

Plant materials, BDMV isolate, and sap-inoculation. *P. vulgaris* (cv. Topcrop) plants were grown for 7 days, and *N. benthamiana* plants were grown for 28 days in a greenhouse (700 to 1,500 μmol m^{-2} s^{-1} photosynthetically active radiation, 30 ± 5.0/22 ± 2.0°C day/night) prior to inoculation. A BDMV isolate from Colombia (BDMV-CO) was used in this study and was maintained in *N. benthamiana* by sap transmission (9,26). Primary leaves of *P. vulgaris* and immature leaves of *N. benthamiana* plants were rub-inoculated with sap prepared from BDMV-infected *N. benthamiana* leaves that were ground in ice-cold, 0.1 M phosphate buffer (pH 8.0). After inoculation, plants were maintained in a controlled environment chamber (250 μmol m^{-2} s^{-1} 16 h per day, 30°C). Six, 12, and 21 days post sap-inoculation (dpi), systemically infected plants were analyzed for the presence and distribution of BDMV DNA by polymerase chain reaction (PCR) and for the cellular/tissue distribution of the virus by immunolocalization.

DNA extraction and PCR analysis. DNA was extracted from approximately 3 to 4 cm^2 of inoculated, immature and mature, systemically infected leaves and from 0.4 to 0.5 g of shoot apex, upper and lower stem, and root tissues using the mini-preparation method of Dellaporta et al. (4) (32). A 10-μl aliquot of the DNA extract was used in a 50-μl PCR amplification mixture as previously described, and degenerate geminivirus primers (PAL1v1978 and PAR1c496) were used to direct the amplification of an approximately 1.1-kbp BDMV DNA-A fragment (32). A 2-μl aliquot of each PCR amplification mixture was analyzed by gel electrophoresis in 0.7% agarose in Tris-borate EDTA buffer, and DNA was visualized under ultraviolet light after staining gels in ethidium bromide. Controls included DNA extracts from mock-inoculated plants and known BDMV-infected plants and water alone.

Tissue fixation and immunolabeling. Plant tissues were excised (1 to 2 mm in width) from inoculated, immature and mature leaves and from the shoot apex, upper and lower stems, and roots of infected and mock-inoculated plants. Tissues were fixed in 0.5% (wt/vol) glutaraldehyde and 1.5% (wt/vol) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 50 mM phosphate buffer (pH 7.0) for 30 min, and then transferred to 1% (wt/vol) glutaraldehyde and 3% (wt/vol) paraformaldehyde in 50 mM phosphate buffer and maintained at 4°C for 5 h. After three 30-min washes in 100 mM phosphate buffer (pH 7.0) at 4°C, the tissue samples were dehydrated through a 20% step-graded ethanol series at 4°C, infiltrated over 2 days through a five-step graded series from 100% (vol/vol) ethanol to 100% (vol/vol) London resin (LR) White (medium grade; Electron Microscopy Sciences), and then embedded in LR white resin. Blocks were polymerized by exposure to a temperature sequence of 37°C for 12 h, 48°C for 12 h, and 60°C for 24 h.

For light microscopy studies, semithin sections (1 to 2 μm thick) were cut with glass knives and dried onto superfrost/plus microscope slides (Fisher Scientific Co., Pittsburgh). Sections were preincubated for 1 h in blocking solution (50 mM Tris-HCl, 150 mM NaCl, 0.1% [vol/vol] Tween 20, and 2% [wt/vol] bovine serum albumin [BSA]). Sections were then transferred directly to 90 μl of appropriate antisera (preimmune or geminivirus capsid protein [CP]) diluted 1:25 in antibody diluent (50 mM Tris-HCl, 150 mM NaCl, 0.1% [vol/vol] Tween 20, 0.1% [wt/vol] BSA, and 0.02% [wt/vol] sodium azide) for 3 h. After four 15-min washes with blocking solution, the sections were incubated for 1 h in secondary antibody (15-nm- or 20-nm-diameter gold-conjugated goat anti-rabbit IgG) diluted 1:40 in antibody diluent. Sections were washed twice for 15 min with blocking solution, followed by two 15-min washes with 50 mM Tris-HCl and 150 mM NaCl buffer, and twice for 15 min with distilled water to remove unbound secondary antibody. Sections were then air-dried and silver-enhanced using a silver-enhancing kit for light and electron microscopy according to the manufacturer's instructions (ICN Biomedicals, Inc., Costa Mesa, CA). The silver-enhanced sections were stained with 0.5% (wt/vol) safranin O for 1 min and observed under a Nikon Optiphoto-2 photo microscope (Nikon Inc., Melville, NY).

For subcellular immunogold localization of BDMV CP, ultrathin sections (60 nm) were cut with a diamond knife and collected on uncoated mesh grids or Formvar film-coated slot grids. The procedures employed for immunolabeling were similar to those described above. After secondary antibody incubation and washes, sections were stained with uranyl acetate (2%, wt/vol) in distilled water and lead citrate for 10 min each, and observed using a Philips EM410LS electron microscope (Philips Electronic Instruments Co., Mahwah, NJ) operated at 80 kV.

Tissue preparation for ultrastructural studies. Plant tissues were excised (2 to 3 mm^2) from systemically infected mature leaves and fixed using a procedure adapted from Park et al. (30). Tissues were incubated at 0°C for 5 h in 2.0% (wt/vol) glutaraldehyde plus 2.0% (wt/vol) paraformaldehyde in 50 mM cacodylate-acetate buffer (pH 7.0). After five 10-min rinses in 100 mM cacodylate-acetate buffer (pH 7.0), tissue samples were post-fixed for 3 h in 1.0% (wt/vol) osmium tetroxide dissolved in distilled water. Following three 10-min rinses in 100 mM cacodylate-acetate buffer (pH 7.0), the tissues were left overnight in buffer. The following day, tissue was rinsed (10 min) in fresh buffer and then transferred for 4 h to a 1.0% (wt/vol) tannic acid solution before washing in distilled water (twice for 30 min) and dehydration through a 10% step-graded series of acetone (0°C); each step was 45 min in duration. Infiltration was achieved over a 2-day period through a five-step graded series into 100% Spurr epoxy resin (Electron Microscopy Sciences) and for a further 7 days in 100% resin. Blocks were polymerized by exposure to a temperature sequence of 37°C for 12 h, 48°C for 12 h, and 70°C for 24 h. Ultrathin sections (60 nm) were cut, stained, and examined as described above.

RESULTS

Detection of BDMV DNA in inoculated plants. Symptoms were first detected in newly emerging leaves of sap-inoculated *P. vulgaris* (first trifoliolate leaves) and *N. benthamiana* approximately 6 dpi; by 12 dpi, symptoms were well developed and included distortion and epinasty of leaves and stunted plant growth (shortened internodes). In both hosts, symptoms were most severe in immature leaves. By 21 dpi, symptoms in both hosts had become noticeably attenuated in newly emerged leaves and developing tissues.

PCR analysis was used to confirm the presence of BDMV DNA in various tissues of systemically infected *P. vulgaris* and *N. benthamiana* plants at 6, 12, and 21 dpi. The degenerate primers PAL1v1978 and PAR1c496 were used, which direct the amplification of a 1.1-kbp fragment from the BDMV DNA-A component (32). By 6 dpi of *P. vulgaris*, BDMV DNA was detected in the shoot apex, both newly emerging trifoliolate leaves, and upper and lower stem tissues of all four plants examined. Viral DNA also

was detected in inoculated leaf tissue of three of four plants and in root tissue of one of four plants. Similar results were obtained for *N. benthamiana* plants at 6 dpi. At 12 dpi, viral DNA was detected in tissues of the shoot apex, newly emerged leaves, and stems of both hosts (Fig. 1). At this time point, viral DNA was still detected in inoculated leaves of both hosts, but less frequently in root tissue (one of seven *P. vulgaris* plants [results of two experiments] and two of six *N. benthamiana* plants [results of two experiments]). At 21 dpi, BDMV DNA was detected in all tissues of both hosts, except in the roots.

No DNA fragments were amplified from extracts of mock-inoculated *P. vulgaris* and *N. benthamiana* plants or water alone, whereas the expected 1.1-kbp DNA fragment was consistently amplified from extracts of known BDMV-infected plants.

These results indicate that, during the systemic infection process, BDMV spreads from rub-inoculated leaves into the shoot apex, newly developed and developing leaves, and stem tissue of *P. vulgaris* and *N. benthamiana* plants by 6 dpi and that infection of these tissues continues at 12 and 21 dpi. Although BDMV DNA had spread into the roots of some plants of both hosts (see below for cellular immunolocalization studies), detection in roots was sporadic and, by 21 dpi, no viral DNA was detected in root tissues of either host.

Evaluation of geminiviral CP antisera for BDMV detection. To identify the cells involved in the establishment of BDMV systemic infection in *P. vulgaris* and *N. benthamiana* plants, we utilized a polyclonal antibody raised in rabbits against an *Escherichia coli*-expressed CP of tomato leaf crumple geminivirus (29), a bipartite geminivirus that is closely related to BDMV (the CPs are 98% identical at the amino acid level). To establish the specificity of this CP antisera, control experiments were performed on tissues from both host species. Semithin sections of BDMV-infected and mock-inoculated tissues were immunolabeled and then silver-enhanced. In both hosts, extensive labeling was observed over the nuclei of the vascular (CC and PP) and BS cells of main veins of systemically infected leaves (Figs. 2A and 3A), whereas no labeling was observed over the nuclei of these cells in comparable leaves from mock-inoculated plants (data not shown). No labeling was observed over the nuclei of cells of infected or mock-inoculated plants when preimmune antisera was used (Figs. 2B and 3B). Weak nonspecific labeling was occasionally detected over the xylem element cell wall with both preimmune and CP antisera (compare Figs. 2A and B and 3A and B).

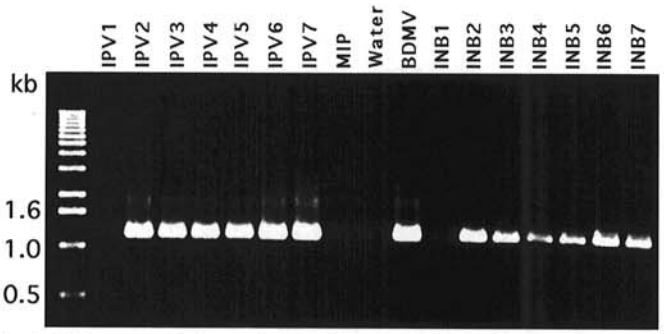

Fig. 1. Polymerase chain reaction amplification of a 1.1-kbp bean dwarf mosaic geminivirus (BDMV) DNA-A fragment from DNA extracts prepared from systemically infected *Phaseolus vulgaris* and *Nicotiana benthamiana* plants, 12 days post sap-inoculation. The primer pair, PAL1v1978 and PAR1c496, was used to direct the amplification of the BDMV DNA-A fragment (32). *P. vulgaris* plant samples IPV1 = root tissue; IPV2 = lower stem below the cotyledonary node; IPV3 = upper stem below the node of the second youngest trifoliolate leaf; IPV4 = inoculated primary leaves; IPV5 = shoot apex; IPV6 = mature second trifoliolate leaf; and IPV7 = immature first trifoliolate leaf. MIP = mature leaves from mock-inoculated plants; Water = DNA extract was replaced by distilled water in the polymerase chain reaction amplification mixture; BDMV = DNA extract from a known BDMV-infected plant. *N. benthamiana* plant samples INB1 = root tissue; INB2 = lower stem below the cotyledonary node; INB3 = upper stem below the node of the second youngest leaf; INB4 = inoculated leaves; INB5 = shoot apex; INB6 = mature second leaf; and INB7 = first immature leaf.

Cellular localization of BDMV in infected *P. vulgaris* plants. To explore BDMV tissue tropism, systemically infected *P. vulgaris* plants were sampled 6, 12, and 21 dpi, and different tissues were immunolabeled with CP antisera. Table 1 presents a synopsis of data collected on the cellular distribution of BDMV as a function of the progression of systemic infection of *P. vulgaris*.

In the inoculated primary leaves (6 and 12 dpi), sparse labeling was consistently observed over the nuclei of MC, BS, CC, and PP cells (Fig. 2H) and also over the cytoplasm of most cell types. Extensive labeling was observed over the nuclei of some PP cells within the major veins of inoculated leaves (data not shown). These results establish that, in inoculated primary leaves of *P. vulgaris*, BDMV can gain access to and replicate in both phloem (PP and CC) and nonphloem (BS, MC, and epidermal) cells.

In the main veins of systemically infected trifoliolate leaves, labeling was consistently detected over the nuclei of phloem cells and BS cells (Fig. 2A, D, and F; Table 1). In some cases, the nuclei of adjacent ground parenchyma cells also gave a positive labeling reaction (Fig. 2A). Nuclei of CC, PP, and BS cells of lower-ordered veins also showed consistent labeling (Fig. 4A to E). In all veins examined, the extent of labeling of these cell types was variable in that not all cells showed nuclear labeling. Labeling also was observed over the nuclei of approximately 11% of the spongy and palisade MC cells examined in systemically infected leaves (Figs. 2D and F and 4A to E), as well as over the nuclei of a small number of epidermal cells in 12-dpi plants (Fig. 4F; Table 1). By 21 dpi, there was a clear reduction in the cell types and overall number of cells in which BDMV was detected in newly developing leaves (third and fourth trifoliolate leaves). BDMV appeared to be restricted to PP, CC, and BS cells in these leaves (Table 1), and some infected phloem cells had collapsed and degraded (data not shown). These findings were consistent with the attenuated symptoms observed in these newly developing leaves.

During these studies, extensive analysis of serial sections revealed a sequential alignment of BDMV-infected cells (i.e., those showing nuclear labeling) from phloem to BS to MC cells (Fig. 4A to E) and, in some cases for 12-dpi plants, out into the epidermal cells. The consistent failure to observe BDMV infection extending out into adjacent cells of the same tissue type from cells along this pathway revealed an unexpected confinement of the virus to these particular groups of cells (Figs. 4A to E and 5).

Immunolocalization experiments conducted on the shoot apex of systemically infected bean plants revealed a complete absence of BDMV in the central zone of the shoot apical meristem. However, extensive labeling was consistently detected over the nuclei of cells of the developing protophloem, which consists of developing PP, CC, and SE cells (Fig. 2C). Here, the CP antigen was most frequently detected in the differentiating PP, as compared with the neighboring differentiating CC (Fig. 2C; Table 1). In the upper stem, BDMV showed a similar tropism for cells of the functional phloem, although it should be noted that BDMV infection also spread out into a few adjacent cortical cells, as well as into the interior of the cambium (Table 1).

Examination of tissues down the plant axis revealed that the number of infected cells (and cell types) decreased markedly from the upper stem to the root (Table 1). Labeling was only detected over the nuclei of a small number of PP and adjacent cambial cells located in the stem beneath the cotyledonary node (Fig. 2E; Table 1). Extensive examination of primary root tissues revealed a paucity of BDMV-infected cells (Fig. 2G), and only on rare occasions was labeling detected over the nuclei of PP cells in these tissues (Fig. 2G; Table 1). Cells with such labeling were not found in the apical tissues of primary and lateral roots (data not shown).

Collectively, these results establish that BDMV infection in *P. vulgaris* is not phloem-limited. However, the virus does show a strong tropism for vascular cell types (PP and CC), as well as for

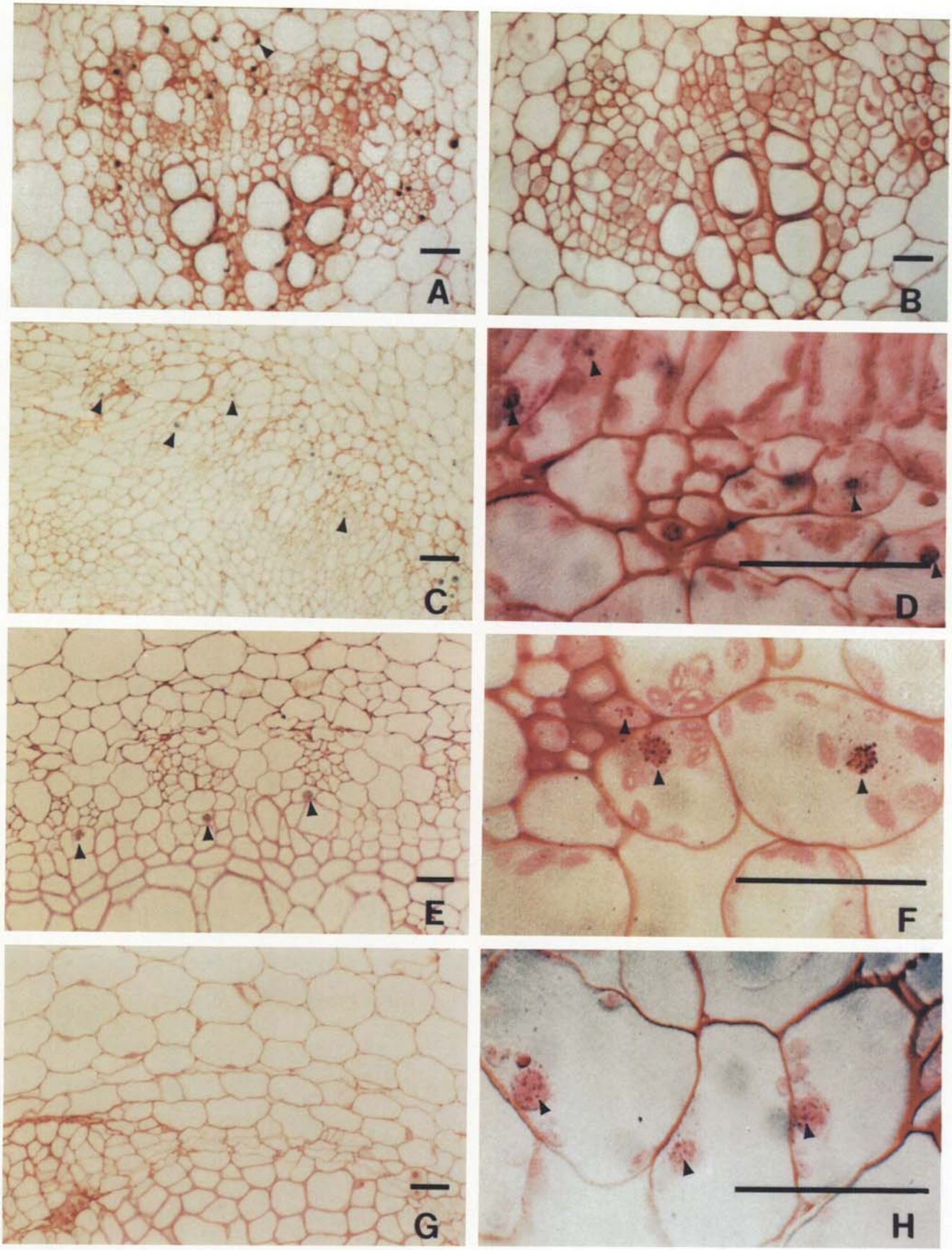

Fig. 2. Cellular localization of bean dwarf mosaic geminivirus (BDMV) by immunolabeling and silver enhancement of the capsid protein (CP) in different organs of systemically infected *Phaseolus vulgaris* plants (12 days post sap-inoculation). Black darts indicate infected cells with immunolabeling over nuclei. Bar = 30 µm. **A,** Transverse section of a main vein in an immature leaf immunolabeled by geminivirus CP antisera. Note the extensive labeling over the nuclei of phloem, bundle sheath, and vein parenchyma cells. **B,** Control; section was similar to **A**, but it was immunolabeled with preimmune antisera. Note the nonspecific binding of the antisera to the xylem element cell wall. The following light micrographs show sections from various tissues immunolabeled with geminivirus CP antisera: **C,** shoot apex; **D,** immature leaves; **E,** stem beneath the cotyledonary node; **F,** mature leaves; **G,** roots; and **H,** inoculated leaves.

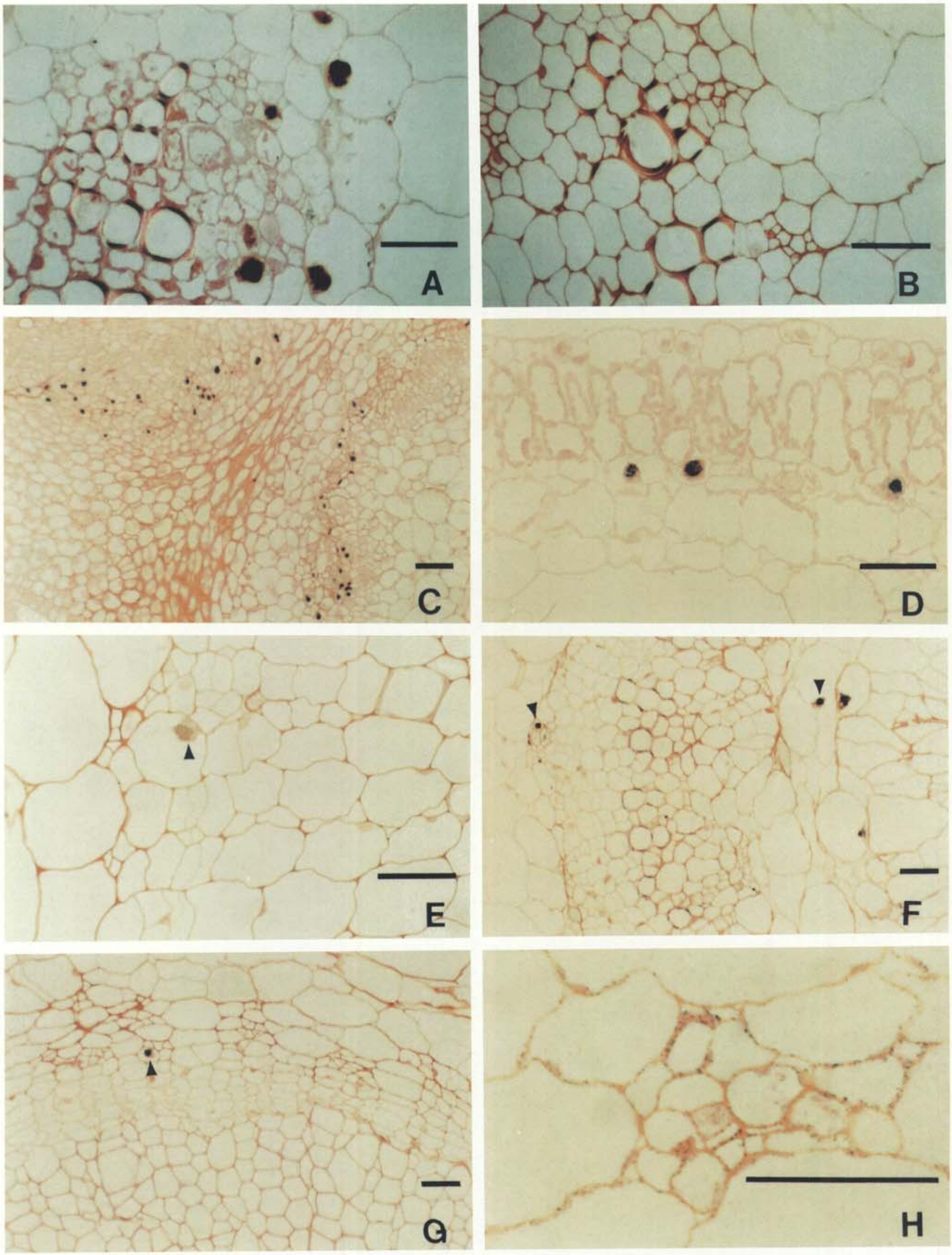

Fig. 3. Cellular localization of bean dwarf mosaic geminivirus (BDMV) by immunolabeling and silver enhancement of the capsid protein (CP) in different organs of systemically infected *Nicotiana benthamiana* plants (12 days post sap-inoculation). Black darts indicate infected cells with immunolabeling over nuclei. Bar = 30 μm. **A,** Transverse section of a main vein in a mature leaf immunolabeled with geminivirus CP antisera. Note the extensive labeling over the nuclei of phloem parenchyma, bundle sheath, and vein parenchyma cells. **B,** Control; section was similar to **A**, but was immunolabeled with preimmune antisera. Note the nonspecific binding of the antisera to xylem element cell wall. The following light micrographs show sections from various tissues immunolabeled with geminivirus CP antisera: **C,** shoot apex; **D,** immature leaves; **E,** stem beneath the cotyledonary node; **F,** mature leaves; **G,** roots; and **H,** inoculated leaves.

the adjacent BS cells. This was particularly the case for infected plants at 21 dpi, in which BDMV infection had become almost limited to these cell types in newly developed and developing tissues (Table 1).

Cellular localization of BDMV in infected *N. benthamiana* plants. A similar set of immunolocalization experiments was performed on tissues prepared from BDMV-infected *N. benthamiana* plants (12 dpi only). In inoculated leaves, labeling was localized over the cytoplasm and nuclei of most cell types within tissues associated with all vein orders; e.g., CC, PP, xylem parenchyma, BS, vascular parenchyma (VP), MC, and epidermal cells (Fig. 3H; Table 2). However, in contrast to the situation observed in *P. vulgaris*, extensive labeling was not observed over the nuclei of any cell type in inoculated leaves (Fig. 3H).

In systemically infected immature leaves, labeling was detected over the nuclei of CC, PP, BS, MC, and epidermal cells (Fig. 3D; Table 2), and the nuclei of some of these cells were enlarged and intensely labeled (Fig. 3D). Examination of serial sections from such tissues revealed a sequential alignment of BDMV-infected cells from phloem to BS to MC cells and, occasionally, out into epidermal cells (Fig. 5; Table 2). This alignment of infected cells was very similar to that observed in infected *P. vulgaris* leaves. Interestingly, in mature leaves, labeling was predominantly observed over the nuclei of CC, PP, and BS cells (Fig. 3A; Table 2). Nuclear labeling was observed in a small number of VP cells of the main vein (Fig. 3F) and only in a few MC adjacent to the BS of the minor veins (data not shown). These results suggest that, compared with immature leaves, viral movement into MC of mature leaves is restricted. The nature of this restriction was not determined, but it could involve degradation of infected phloem cells such as that observed in *P. vulgaris* at 21 dpi or structural differences in the vasculature of mature versus immature leaves or both.

As observed in infected *P. vulgaris* plants, BDMV was not detected in the shoot apical meristem of systemically infected *N. benthamiana* plants, but extensive labeling was consistently detected over the nuclei of PP and CC in the protophloem of the shoot apex (Fig. 3C; Table 2). In contrast to *P. vulgaris*, the nuclei of PP and CC of BDMV-infected *N. benthamiana* protophloem tissue displayed an almost equal extent of labeling (Fig. 3C; Table 2). Outside of the protophloem, labeling was observed over the nuclei of a small number of VP, cortical, and pith cells. Examination of the plant axis revealed a sharp decrease in the number and type of cells infected in stem and root tissues (Tables 1 and 2). Labeling was only observed over the nuclei of some CC, PP, cambial, and VP cells of stem tissues located beneath the cotyledonary node (Fig. 3E; Table 2). In primary root tissue of infected *N. benthamiana* plants, the presence of the virus was detected in only a limited number of PP and neighboring cambial cells (Fig. 3G; Table 2). No infected cells were detected in the apical tissues of primary or lateral roots (data not shown).

These results establish that BDMV infection in *N. benthamiana* is not phloem-limited but, as observed in infected *P. vulgaris* plants, the virus does show a strong tropism for vascular cell types (PP, CC), as well as for the adjacent BS cells. Overall, the cell and tissue types infected by BDMV in *N. benthamiana* plants at 12 dpi were similar to those infected in *P. vulgaris* plants at the same time point.

Subcellular localization of BDMV in infected *P. vulgaris* and *N. benthamiana* plants. The subcellular location of the CP antigen was next investigated to confirm the presence of BDMV in nonphloem cell types. Previous ultrastructural studies have established that the nuclei of geminivirus-infected cells become swollen and display nucleolar segregation, fibrillar rings, and large granular inclusion bodies composed primarily of virus-like particles (15–19,22,26,31,33). Ultrastructural studies performed on BDMV-infected tissues from inoculated and systemically infected leaves revealed the presence of similar cytopathological characteristics (Fig. 6A). No tubule-like structures (18) were observed in BDMV-infected tissues.

TABLE 1. Analysis of bean dwarf mosaic geminivirus (BDMV) distribution in systemically infected *Phaseolus vulgaris* plants at 6, 12, and 21 days post sap-inoculation (dpi) of the primary leaf based on immunolocalization studies using capsid protein antisera[a]

	Axis system					Leaf system[b]				
Cell types	Shoot apex	Upper stem	Lower stem	Root	Cell types	1st TL	2nd TL	3rd TL	4th TL	Inoculated
Companion cell	••[c]	••	•	–	Companion cell	•	•			•
	♦♦	♦♦	♦	–		♦♦	♦♦			♦
	#	#				#	##	#	#	
Phloem parenchyma	•••••	•••••	••	–	Phloem parenchyma	•••	•••			••
	♦♦♦♦♦♦	♦♦♦♦	♦♦	♦		♦♦♦♦	♦♦♦			♦♦
	##	##				##	##	##	##	
Cambium	•	–	–	–						
	♦	♦	♦♦	–						
	–	–								
Xylem parenchyma	–	–	–	–	Xylem parenchyma	–	–			•
	–	–	–	–		–	–			♦
	–	–				–	–	#	#	
Vascular parenchyma	••	•••	•	–	Vascular parenchyma	••	••			••
	♦♦♦	♦♦♦	♦	–		♦♦	♦♦			♦♦
	#	#				#/–	#	##	#	
Cortex	•	••	–	–	Bundle sheath	•••	•••			•••
	♦♦	♦♦♦	–	–		♦♦♦	♦♦♦♦			♦♦
	–	–				#	#	##	##	
Pith parenchyma	•	•	–	–	Mesophyll	•••	••			••
	–	♦	–	–		♦♦♦	♦♦♦♦			♦♦♦
	–	–				–	#/–	##	##	
Epidermal	–	–	–	–	Epidermal	–	–			••
	–	–	–	–		♦	♦			♦
	–	–				–	–	–	–	

[a] The location of the specific tissue analyzed is illustrated in Figure 5.

[b] The primary leaves were inoculated; numbers represent trifoliolate leaves (TL) from youngest to oldest (the 1st TL of 6-dpi plants corresponds to the 4th TL of 21-dpi plants).

[c] Immunolocalization of BDMV capsid protein is represented by •, ♦, and # that indicate the presence of the virus within cells of *P. vulgaris* plants at 6, 12, and 21 dpi, respectively. The number of symbols indicates the relative number of cells of each type that were labeled. Cells in which labeling was not detected are indicated by –.

The specificity of the geminivirus CP antisera for immunolocalization studies was established by comparing preimmune and immune (CP) antisera (Fig. 6B and C). In infected *P. vulgaris* and *N. benthamiana* plants, similar subcellular patterns of CP distribution were detected and in a pattern that was consistent with results of the light microscopy studies. Most labeling was detected over the nuclei of cells with large nuclear inclusion bodies, and nuclear inclusion bodies and associated heavy labeling were detected in phloem and nonphloem cells (Fig. 6C to E). In the absence of nuclear inclusion bodies, labeling of nuclei was reduced to a level equivalent to that detected with preimmune antisera. Sparse labeling was detected over the cytoplasm of infected cells, regardless of whether the nuclei contained inclusion bodies. Occasionally, labeling was detected over chloroplasts (Fig. 6F) and mitochondria (data not shown), but the level of labeling was only marginally above background. Labeling at background levels was observed over SEs in inoculated or systemically infected mature leaves and in the shoot apex.

DISCUSSION

The PCR-based analysis of the distribution of BDMV DNA within infected *P. vulgaris* and *N. benthamiana* plants revealed that, by 6 dpi, the virus had spread from inoculated leaves to cells of the shoot apex, newly developed and developing leaves, and the stem and primary root tissues. The sporadic detection of viral

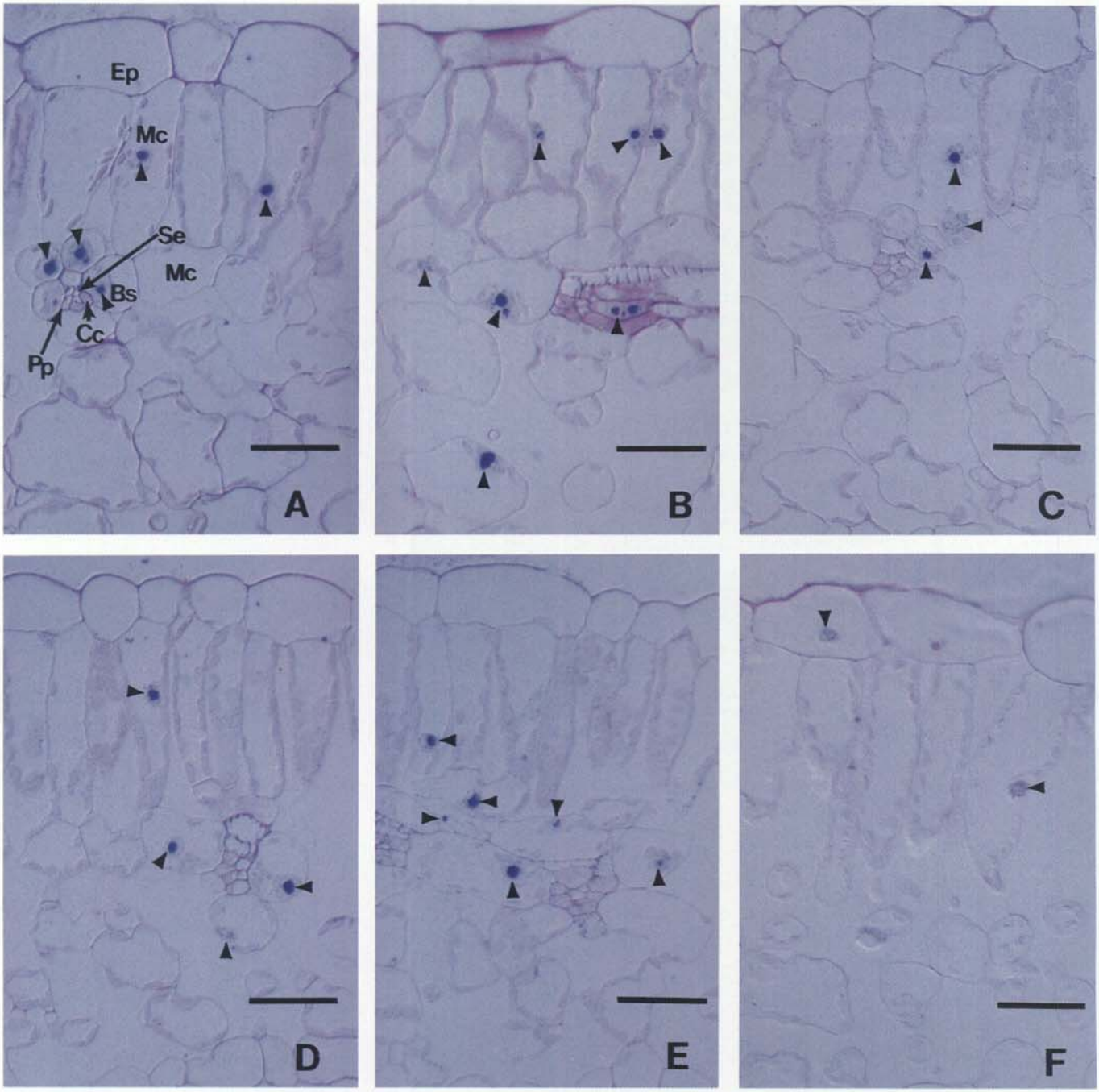

Fig. 4. Cellular localization of bean dwarf mosaic geminivirus (BDMV) by immunolabeling and silver enhancement of the capsid protein (CP) in sections from leaves of systemically infected *Phaseolus vulgaris* plants (12 days post sap-inoculation) showing the nonphloem infection domain consisting of sequentially aligned infected bundle sheath (Bs) and mesophyll cells (Mc). Black darts indicate infected cells with immunolabeling over nuclei. Bar = 30 μm. The following light micrographs show sections from different systemically infected leaves immunolabeled with geminivirus CP antisera including **A to E,** sequential infection of Bs and Mc cells; and **F,** section showing labeling over the nucleus of an infected epidermal (Ep) cell. Cc, companion cell; Pp, phloem parenchyma; and Se, sieve element.

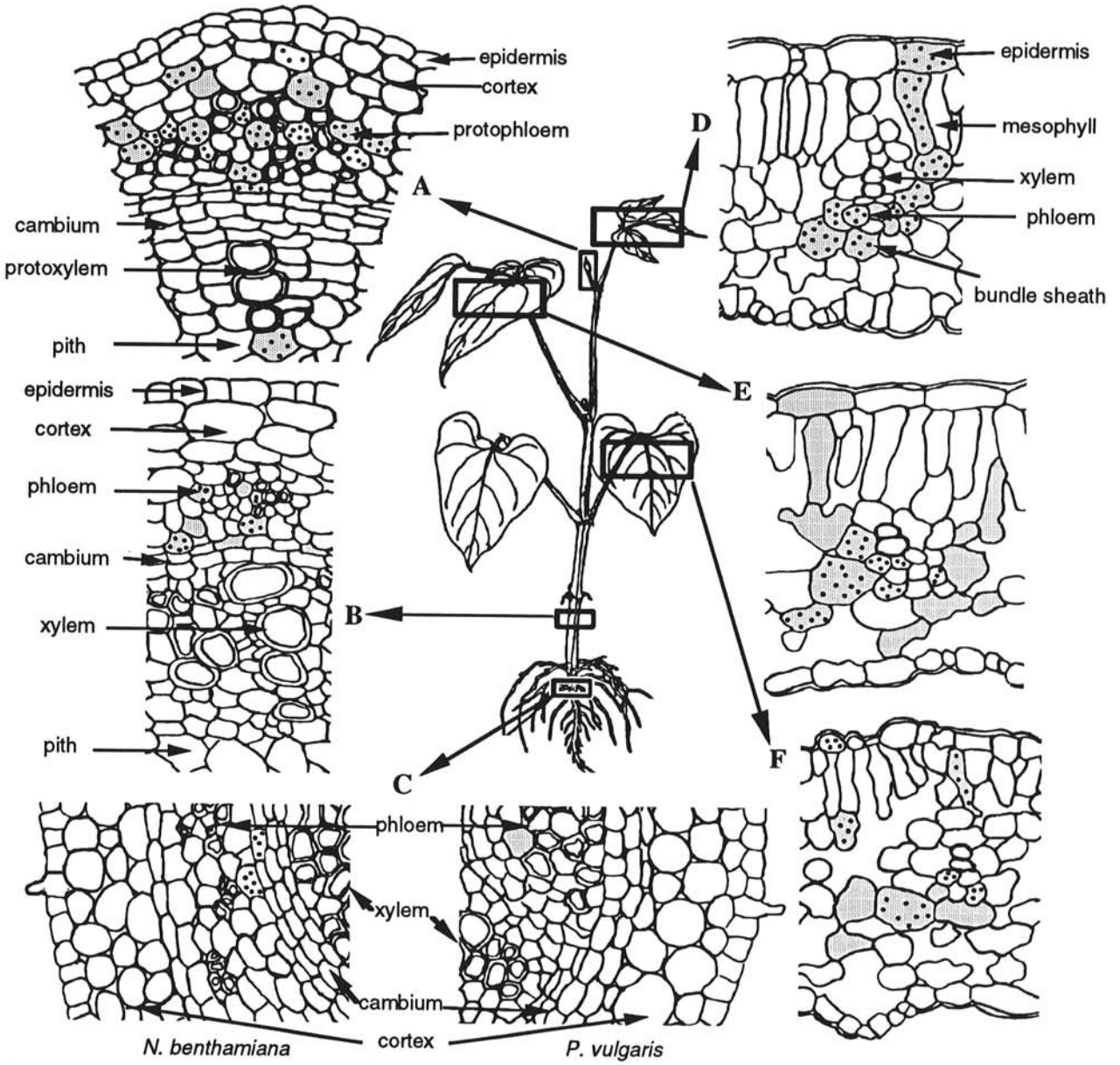

Fig. 5. Schematic representation of infection domains of bean dwarf mosaic geminivirus (BDMV) in inoculated *Phaseolus vulgaris* and *Nicotiana benthamiana* plants, 12 days post sap-inoculation with BDMV. Cells indicated in gray (*P. vulgaris*) or with black dots (*N. benthamiana*) represent sites of BDMV infection. The basis for the identification of the infection domain concept comes from careful observations carried out on over 5,000 sections (including semi-serial) obtained from BDMV-infected tissues.

TABLE 2. Analysis of bean dwarf mosaic geminivirus (BDMV) distribution in systemically infected *Nicotiana benthamiana* plants at 12 days post sap-inoculation (dpi) of immature leaves based on immunolocalization studies using capsid protein antisera[a]

	Axis system				Leaf system[b]		
Cell types	Shoot apex	Lower stem	Root	Cell types	1st SL	2nd SL	Inoculated
Companion cell	♦♦♦♦♦[c]	♦	–	Companion cell	♦♦	♦♦	♦
Phloem parenchyma	♦♦♦♦♦♦	♦	♦	Phloem parenchyma	♦♦♦	♦♦♦	♦
Cambium	♦	♦	♦				
Xylem parenchyma	–	–	–	Xylem parenchyma	–	–	♦
Vascular parenchyma	♦♦♦	♦	–	Vascular parenchyma	♦♦♦	♦♦	♦♦
Cortex	♦♦	–	–	Bundle sheath	♦♦♦	♦♦	♦
Pith parenchyma	♦	–	–	Mesophyll	♦♦♦	♦	♦♦
Epidermal	–	–	–	Epidermal	♦	–	♦

[a] The location of the specific tissue analyzed is illustrated in Figure 5.
[b] The immature leaves were inoculated at the four-leaf stage; numbers represent systemic leaves (SL) from youngest to oldest.
[c] Immunolocalization of BDMV capsid protein is represented by ♦ that indicates the presence of the virus within cells of *N. benthamiana* plants 12 dpi. The number of symbols indicates the relative number of cells of each type that were labeled. Cells in which labeling was not detected are indicated by –.

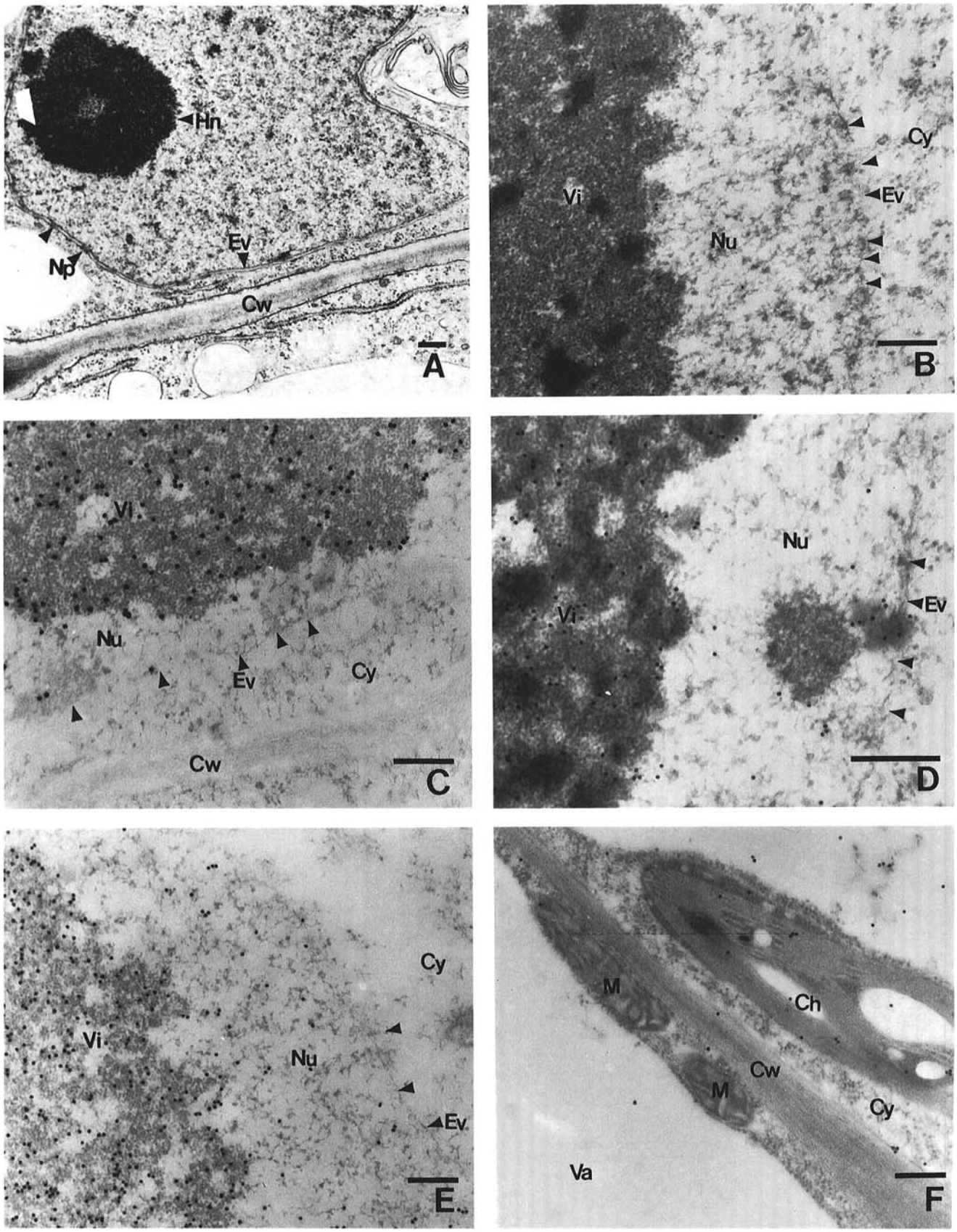

Fig. 6. Subcellular localization by immunogold labeling of bean dwarf mosaic geminivirus (BDMV) capsid protein (CP) in different cells types of systemically infected *Phaseolus vulgaris* plants (12 days post sap-inoculation). Bar = 300 nm. **A,** Electron micrograph of a section cut from an infected mature leaf sample showing the sharply segregated nucleolus of a phloem parenchyma cell. Note the plasma membrane, nuclear envelope, and the envelope pores. **B,** Electron micrograph of the nucleus of an infected phloem parenchyma cell in the shoot apex labeled with preimmune antisera. The following electron micrographs show ultrathin sections immunolabeled with geminivirus CP antisera: **C,** nuclei of an infected phloem parenchyma cell in the minor vein of an immature leaf and **D,** in the shoot apex; **E,** nucleus of an infected mesophyll cell in a mature leaf and **F,** infected bundle sheath cell in an inoculated leaf. Ch, chloroplast; Cw, cell wall; Cy, cytoplasm; Ev, nuclear envelope; Hn, hypertrophied nucleolus; M, mitochondrion; Np, nuclear pore; Nu, nucleoplasm; Va, vacuole; and Vi, viral inclusion body.

DNA in root tissues suggests that virus movement was directed toward aboveground versus belowground plant parts, which probably reflects the relative sink strength of the shoot apex. In both hosts, the tissue distribution of BDMV, as inferred by detection of the viral CP antigen, was similar to that established for the viral DNA. These results suggest that tissues and cells in which CP was detected were actually infected by BDMV, as opposed to only having virus-independent accumulations of CP. A second line of evidence supporting this conclusion is the finding that *E. coli*-expressed tomato leaf crumple geminivirus CP microinjected into *P. vulgaris* or *N. benthamiana* MC cells is unable to independently move cell-to-cell or increase the SEL of plasmodesmata (M. R. Rojas, V. P. Patel, W. J. Lucas, and R. L. Gilbertson, *unpublished data*).

The cells and cell types that were infected by BDMV within the tissues examined in this study are illustrated in Figure 5. In both hosts, BDMV infected nonphloem cells in inoculated and systemically infected leaves. Thus, BDMV is a bipartite geminivirus that is not phloem-limited in a natural host (*P. vulgaris*) or an experimental host (*N. benthamiana*). This finding is consistent with the high efficiency (approximately 100%) with which BDMV is sap-transmitted to both host plants. The presence of the BDMV in most cell types in inoculated leaves suggests that the virus probably reaches the vascular system of these leaves via cell-to-cell movement from initially infected MC or epidermal cells or both, as opposed to being directly introduced via sap-inoculation into phloem cells or sieve elements. This suggestion is further supported by the finding that, in systemically infected leaves, the virus can exit from infected phloem cells and spread, cell-to-cell, into adjacent BS and associated MC and epidermal cells.

The unexpected finding that BDMV infection was restricted to vascular (PP and CC) and BS cells of newly developing leaves of *P. vulgaris* plants at 21 dpi suggests that the developmental stage of the plant may have an important influence on the ability of BDMV to spread into certain cell types. This might also explain why successful sap-transmission of BDMV requires inoculation of plants at an early stage of development (e.g., at the one-half to three-quarters expanded primary leaf stage for *P. vulgaris*). The host (developmental?) factors responsible for this restriction of the BDMV infection have yet to be identified. However, the attenuated symptoms observed in these leaves taken together with the finding that phloem-associated cells (SE, CC, and PP) in such BDMV-infected tissues were undergoing cellular degradation raises the possibility that this restriction could be because of a host defense response.

Previous studies have established that some whitefly-transmitted geminiviruses can exit the phloem in infections of experimental hosts (17,22,27,33). However, in studies in which natural hosts were examined, whitefly-transmitted geminiviruses were reported to be phloem-limited (10,13,15,16,19,22,31,36). As these studies were based almost entirely on ultrastructural examination of tissues selected from infected plants at a single time point, the possibility exists that infection of a small number of nonphloem cells could have gone undetected or that the developmental stage of the plant used in these studies may have been comparable with the 21-dpi time point in *P. vulgaris*, in which BDMV infection was restricted to phloem and BS cells. Reexamination of the question of phloem limitation in the natural hosts of whitefly-transmitted geminiviruses using highly sensitive detection methods and time course studies may provide a more accurate indication of the true extent of phloem limitation of these viruses. Given that many whitefly-transmitted geminiviruses are sap-transmissible, it seems likely that many of these viruses may not be phloem-limited, particularly early in the infection process.

Two BDMV infection domains (defined here as a group of specific host cell types in which a virus can replicate and move) were identified in the present study: a phloem domain that includes the protophloem, mature phloem, and associated vascular cells and a nonphloem domain that extends out from infected phloem cells to include sequentially aligned BS, MC and, to a lesser extent, epidermal cells. Although the present study established that BDMV can exit from phloem cells and infect nonphloem cell types, viral infection always was closely associated with the phloem. This was most evident in the extensive infection of the protophloem cells within the developing shoot apex (Figs. 2C and 3C; Tables 1 and 2). This is perhaps not surprising, because the protophloem is the tissue that eventually gives rise to the functional phloem cells (PP and CC) that are most commonly infected by whitefly-transmitted geminiviruses. In general, infection of protophloem cells is probably a critical early step in the systemic infection of plants by whitefly-transmitted geminiviruses. The near confinement of BDMV to cells of the protophloem and the exclusion of the virus from the true apical meristem reveals an infection domain that may well reflect a limitation imposed on virus movement through plasmodesmata (23) or rapid cell division within the apical meristem or both.

In systemically infected leaves, the nonphloem infection domain involved predominantly BS and MC cells and, occasionally, epidermal cells that were sequentially aligned such that they appeared to form sectors emanating from infected SE-CC cells (Figs. 4A to E and 5D and E). The underlying basis for the establishment of this infection domain may well reflect the presence of endogenous macromolecular communication domains within the leaf, involving plasmodesmata. Support for this hypothesis is provided by recent chlorophyll fluorescence studies performed on source leaves (1,35), which revealed the presence of similar domains.

Based on the present findings, a model of systemic BDMV infection in sap-inoculated *P. vulgaris* and *N. benthamiana* plants can be advanced (it should be noted here that BDMV infection in *P. vulgaris* mediated by the whitefly vector in nature may not show an identical pattern of virus distribution). Virions are introduced into wounded epidermal and MC cells during sap-inoculation and, within these cells, initial viral replication takes place. Within the inoculated leaves, the infectious form(s) of the virus (referred to hereafter as the virus) moves cell-to-cell from these infected epidermal and MC cells and accesses BS, PP, and CC cells. From infected PP and CC cells, the virus enters the SE for long-distance transport to sink tissues, in particular the shoot apex (strong sink). Upon delivery to the shoot apex, the virus exits from functioning SEs and accesses developing cells of the protophloem, presumably via the interconnecting plasmodesmata. The infection of protophloem cells ensures that, as a leaf develops and becomes a photoassimilate source, the virus can infect mature PP and CC cells and eventually reenter SEs to move to new sink tissues, thereby resulting in a progressive infection cycle. The sporadic distribution of virus within the mature phloem cells of the minor veins of systemically infected leaves likely reflects whether the progenitor cells of these cells were infected by BDMV in the protophloem of the shoot apex. Finally, in systemically infected leaves, BDMV exits from infected mature phloem cells into BS, MC, and epidermal cells; this may depend upon the developmental stage of the host.

The demonstration that BDMV infects MC cells of both host plants provides unequivocal support for the previous conclusions regarding cell-to-cell movement of BDMV, which were based on microinjection experiments performed on MC cells (28). The finding that BDMV infects MC cells in systemically infected *N. benthamiana* and the absence of BDMV from the true meristem is consistent with results of a recent study of tomato golden mosaic geminivirus infection in *N. benthamiana* (27). Future studies will employ advanced confocal microscopy methods to further our understanding of the developmental and physiological basis for BDMV infection domains identified in this study.

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