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Cellular Basis of Potato Spindle Tuber Viroid Systemic Movement

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Viroids are small, nontranslatable pathogenic RNAs that replicate autonomously and traffic systemically in their host plants. We have used *in situ* hybridization to analyze the trafficking pattern of *Potato spindle tuber viroid* (PSTVd) in tomato and *Nicotiana benthamiana*. When PSTVd was inoculated onto the stem of a plant, it replicated and trafficked to sink, but not source, leaves. PSTVd was absent from shoot apical meristems. In the flowers of infected plants, PSTVd was present in the sepals, but was absent in the petals, stamens, and ovary. The replicative form of PSTVd was detected in the phloem. Our data demonstrate that (i) PSTVd traffics long distance in the phloem and this trafficking is likely sustained by replication of the viroid in the phloem, and (ii) PSTVd trafficking is governed by plant developmental and cellular factors. The dependency of PSTVd and other viroids on cellular mechanisms for RNA trafficking makes them excellent tools to study such mechanisms.

INTRODUCTION

Viroids are single-stranded, covalently closed, circular, pathogenic RNAs that infect plants (Diener, 1979; Semancik, 1979; Riesner and Gross, 1985). Their genomes range in size from 246 to 399 nucleotides. Although they do not encode any proteins, viroids replicate autonomously and traffic systemically throughout their hosts. This is in sharp contrast to viruses which encode proteins that interact with a host plant to accomplish functions ranging from replication to systemic movement (Diener, 1979; Matthews, 1991). The simplicity of a viroid genome and the fact that this RNA genome must interact directly with its host components to accomplish various functions for infection make viroid infection a unique and attractive model system with which to investigate biological processes such as RNA trafficking in a plant.

Generally speaking, the systemic infection process of a viroid can be divided into two major phases: replication in individual plant cells and movement throughout the whole plant. Many viroids replicate in the nucleus (Semancik *et al.,* 1976; Schumacher *et al.,* 1983; Diener, 1979; Harders *et al.,* 1989; Bonfiglioli *et al.,* 1994, 1996). Systemic movement of *Potato spindle tuber viroid* (PSTVd) and other viroids that replicate in the nucleus consists of the following distinct, but related steps (Ding *et al.,* 1999): (1) import into the nucleus through nuclear pores (prior to replication), (2) export out of the nucleus (after replication), (3) cell-to-cell movement, and (4) long-distance movement. Nuclear import of PSTVd appears to be a specific and carrier-mediated process (Woo *et al.,* 1999). Cell-to-cell movement of PSTVd occurs through plasmodesmata and appears also to be mediated by cellular factors (Ding *et al.,* 1997). Based on the observation that, at the whole plant level, PSTVd infection in tomato spreads in a pattern similar to photoassimilate transport, Palukaitis (1987) suggested that PSTVd moves long distance through the phloem. Consistent with this hypothesis, Hammond (1994) and Stark-Lorenzen *et al.* (1997) have shown by *in situ* hybridization that PSTVd is localized in the vascular as well as surrounding tissues of the stems and roots of infected tomato plants. Higher resolution studies have shown that two other viroids, *Coconut cadang cadang viroid* and *Citrus exocortis viroid,* are also localized to the vascular and mesophyll tissues of infected plants (Bonfiglioli *et al.,* 1996).

We have undertaken a comprehensive analysis, by *in situ* hybridization, of the patterns of systemic trafficking of PSTVd in tomato and *Nicotiana benthamiana*. In this paper, we present high-resolution cytological as well as experimental evidence that PSTVd traffics long distance through the phloem, that PSTVd replicates actively in the phloem, and that PSTVd trafficking is governed by certain developmental and cellular parameters. The implications of these findings are discussed.

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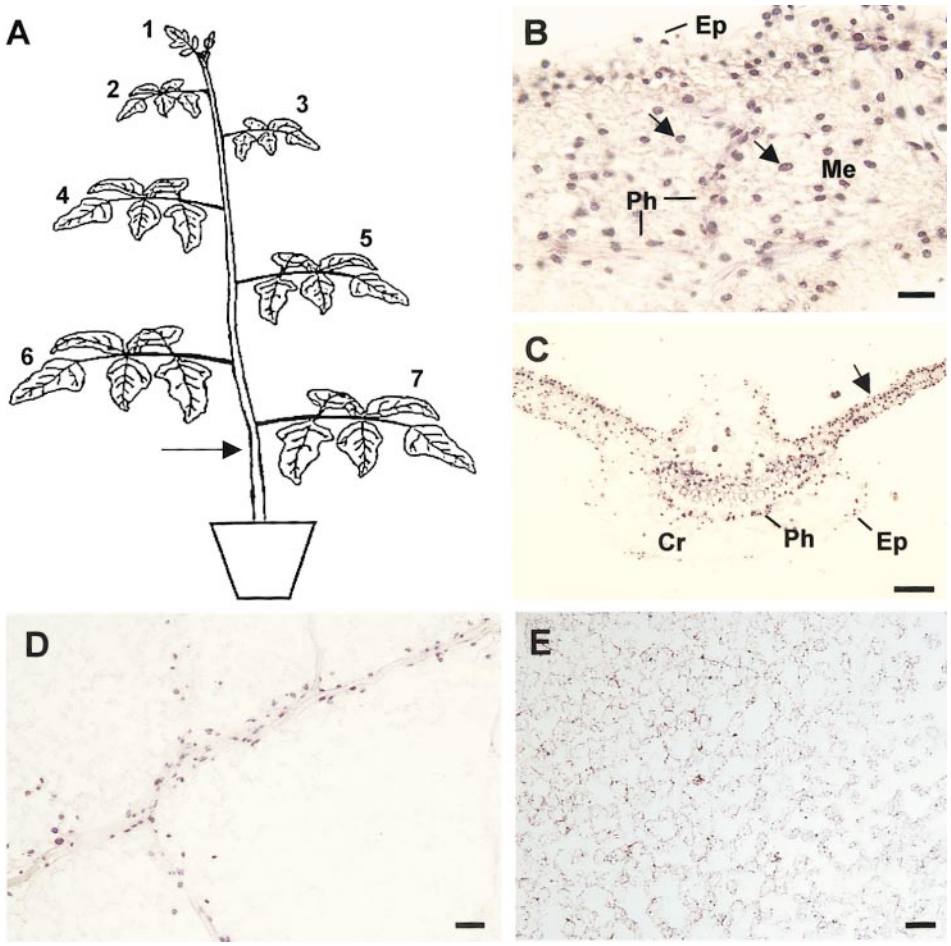

FIG. 1. PSTVd trafficking as a function of leaf development in infected tomato. (A) An idealized view of a tomato plant with leaves at different developmental stages. Leaves 1 and 2 are photosynthetic sinks, leaf 3 is a transition leaf, and leaves 4, 5, 6, and 7 are source leaves. Arrow indicates where *A. tumeficiens* harboring PSTVd cDNA was inoculated onto the plant. (B) Oblique paradermal section of a sink leaf showing presence of PSTVd in phloem (Ph), mesophyll (Me), and epidermal (Ep) cells. The viroid is concentrated in the nuclei of infected cells (arrows). Bar = 10 μm. (C) Transverse section of the petiole of a sink leaf, showing presence of PSTVd in the cortical cells (Cr) as well as in the phloem (Ph). The viroid is also visible in some epidermal cells (Ep). In the leaf blade (arrow), the viroid is present in all types of cells. Bar = 40 μm. (D) Paradermal section of a leaf undergoing sink–source transition. PSTVd is confined mostly to the vascular tissue. Bar = 15 μm. (E) Paradermal section of a source leaf showing absence of PSTVd from all cells. Bar = 15 μm.

RESULTS

PSTVd traffics into sink, but not source, leaves

To gain an understanding of the role of plant development in RNA trafficking, we conducted experiments to determine how the transition of a young leaf from photosynthetic sink to source would affect PSTVd systemic movement. We first performed carboxyfluorescein (CF) translocation studies to determine the sink or source status of leaves in a noninfected tomato. CF has been shown to be transported in the same manner as are photoassimilates and its translocation pattern can therefore mark sink and source leaves. Basically, young leaves that import and unload CF are sinks for photoassimilates, and mature leaves that do not import CF are sources for photoassimilates (Roberts *et al.,* 1997; Oparka *et al.,* 1999; Itaya *et al.,* 2000). Once we determined the sink and source status of leaves in a tomato plant (data not shown), we inoculated a part of the stem below all leaves with PSTVd cDNA via *Agrobacterium*-mediated inoculation (Hammond, 1994) (Fig. 1A). The *A. tumefaciens* strain used as the inoculum contained a binary vector carrying a full-length cDNA of the intermediate strain of PSTVd under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Hu *et al.,* 1997). Primary PSTVd RNA transcripts generated by the activity of the CaMV 35S promoter are capable of initiating natural PSTVd RNA–RNA replication (Wassenegger *et al.,* 1994; Hu *et al.,* 1997).

Three weeks after inoculation, tomato leaves were examined for the presence of PSTVd RNA by dot-blot analysis (data not shown). CF translocation experiments were repeated on infected plants to confirm that, at the time leaf samples were taken for analysis, source leaves

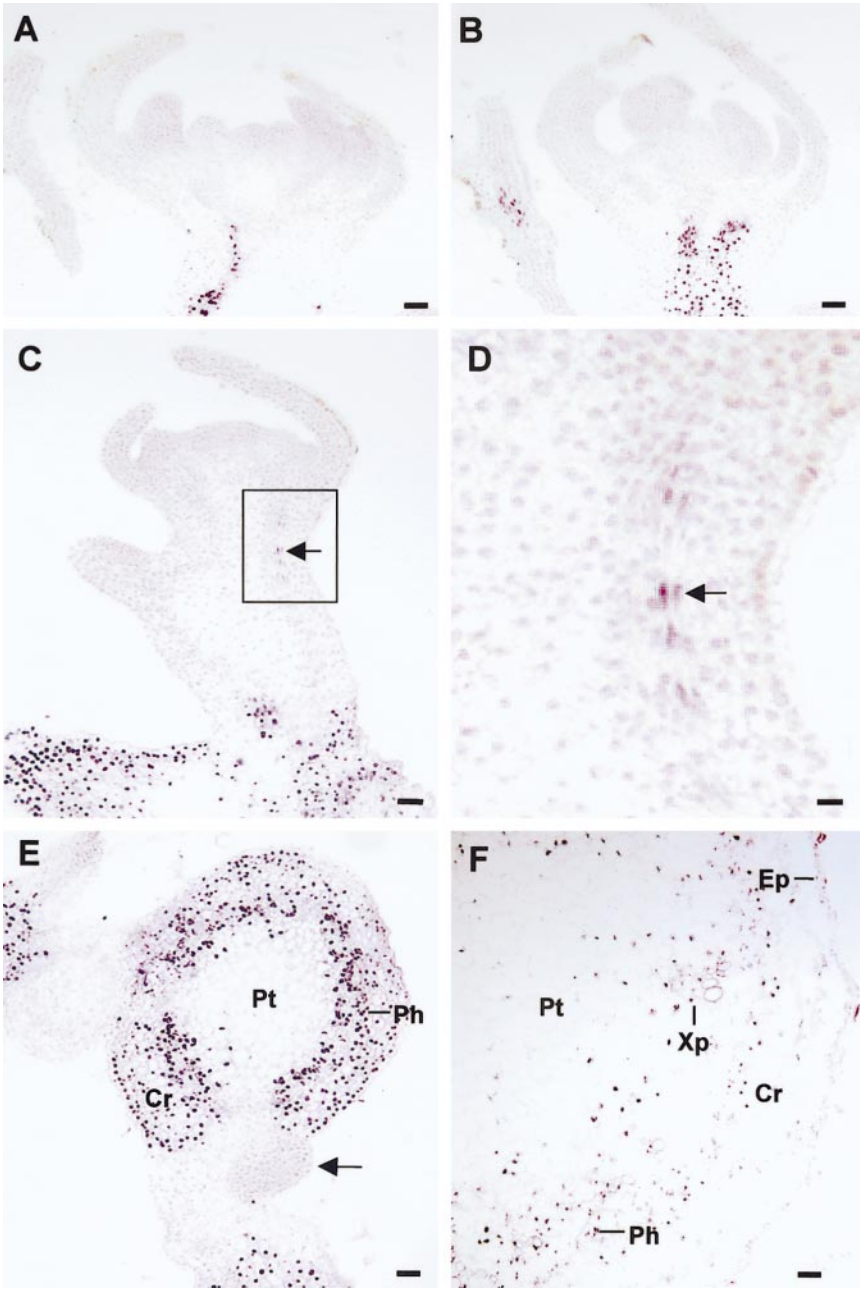

FIG. 2. PSTVd in the vegetative shoot apex and stem of infected *N. benthamiana*. (A, B) Serial longitudinal sections of shoot apex. Note the absence of PSTVd from the apical meristem and its presence in the subapical protophloem. (A) PSTVd in a single vascular bundle and (B) a glancing view of perhaps several vascular bundles containing PSTVd. Bar = 30 μm. (C, D) Longitudinal section from a lateral shoot branch. Note the absence of PSTVd in meristem. The viroid is present in what appears to be the procambium (arrow) and in all cells distal to the meristem. The area within the rectangle in (C) is enlarged in (D). Bar in (C) = 20 μm. Bar in (D) = 5 μm. (E) Transverse section of subapical region showing PSTVd invasion of cortical (Cr) and pith (Pt), in addition to the phloem (Ph). Arrow indicates an axillary bud free of PSTVd. Bar = 20 μm. (F) Transverse section of a portion of stem showing PSTVd in all parenchymatous cell types. Ep, epidermis, Cr, cortex, Ph, phloem, Xp, xylem parenchyma, Pt, pith. Bar = 20 μm.

determined at the time of inoculation remained as source leaves. During this period, some leaves that were sinks at the time of inoculation had matured to become new source leaves, and others were undergoing sink–source transition. The youngest leaves (i.e., those emerging after inoculation) were sinks at the time of leaf sampling. Different leaves were processed for *in situ* hybridization to determine the cellular localization pattern of the predominant, plus-sense PSTVd RNA.

As shown in Fig. 1B, the viroid was present in the phloem, mesophyll, and epidermal cells of a sink leaf. In transverse sections of petioles from sink leaves, PSTVd was also found in cortical cells (Fig. 1C). In every infected cell, PSTVd was mostly localized in the nucleus,

consistent with previous findings (Harders *et al.,* 1989; Woo *et al.,* 1999). In a leaf undergoing sink–source transition, PSTVd was overwhelmingly confined to the vascular tissue (Fig. 1D). In leaves that were sources at the time of inoculation, PSTVd was absent from all cell types 3 weeks postinoculation (Fig. 1E). Data indicate that PSTVd did not move into a source leaf. Once the viroid moved into a sink leaf, however, it remained to be detectable when the leaf developed into a source (data not shown). In these and all following experiments, noninfected tissues were also analyzed using the same protocol. In all cases, no hybridization signals were observed (data not shown).

PSTVd does not invade the shoot apical meristem

Shoot apical meristems (SAM) of mechanically inoculated *N. benthamiana* plants did not contain PSTVd (Figs. 2A and 2B). The viroid, however, was present in the vascular tissues, most likely the procambium and/or protophloem, immediately below the SAM (Figs. 2A and 2B). This observation was confirmed by examining serial longitudinal sections. The viroid was also absent from lateral shoot meristems (Fig. 2C). In favorable sections, hybridization signals could be seen in what appeared to be a procambial region lacking mature phloem cells (Fig. 2D). Data suggested that PSTVd had strong vascular tropism immediately below the SAM. As cells became more differentiated further away from the SAM, the viroid escaped the phloem and began to invade neighboring cells such as the cortical and pith cells (Fig. 2E). In the stem, the viroid was present in all parenchymatous tissues including the epidermis, cortex, phloem, xylem parenchyma, and pith (Fig. 2F). Essentially the same infection pattern was observed in mechanically inoculated tomato (data not shown).

PSTVd is present in sepals, but not in other floral parts

We examined serial sections of infected floral organs of both *N. benthamiana* and tomato for the presence of PSTVd. Figure 3A illustrates a longitudinal view of a tomato flower. In developing flowers of *N. benthamiana* (Figs. 3B and 3C) and tomato (data not shown), PSTVd was detected in the vascular cells, but not in other cells of sepals. It was absent in all cells of the petals, stamens, style, and ovary. The viroid was consistently present in the vascular cells just below the specific floral parts, as revealed by serial longitudinal sections (Figs. 3B and 3C). In more mature flowers of tomato (Fig. 3D–3G) and *N. benthamiana* (data not shown), PSTVd was present in all parenchymatous cells of sepals, but not in any cells of petals, stamens, style, or ovary. This was surprising in light of the fact that phloem connections were already fully established in such developed flowers (Figs. 3H and 3I).

PSTVd replicates in the phloem

Presence of PSTVd in the nuclei of the phloem parenchyma/companion cells could be due to either replication of PSTVd in these cells or import of PSTVd RNA synthesized in nearby nonvascular cells. To test whether PSTVd would replicate in the phloem, we used *in situ* hybridization to look for the presence of the minus-strand RNAs found in PSTVd replicative intermediates. Although present at low levels (i.e., ~1% of that of the plus strand; Harders *et al.,* 1989), minus-strand PSTVd RNA was clearly detectable in the nuclei of phloem parenchyma and companion cells (Fig. 4). All our analyses were performed under high-stringency conditions designed to eliminate hybridization between plus-strand PSTVd molecules (Harders *et al.,* 1989). These data strongly suggested that PSTVd replicates actively in the phloem during long-distance movement.

DISCUSSION

We have used *in situ* hybridization to directly demonstrate that PSTVd traffics long distance through the phloem of infected host plants, thereby providing evidence at the cellular level to support the hypothesis of Palukaitis (1987). The observation that PSTVd traffics into sink but not source leaves during systemic movement suggests that viroid movement follows the pattern of photoassimilate and viral movement in this regard (Leisner and Turgeon, 1993; Roberts *et al.,* 1997). Interestingly, systemic movement of the nucleotide sequence-specific signals for gene silencing also appears to follow this pattern (Voinnet *et al.,* 1998).

In an early study, Momma and Takahashi (1983) showed that *Hop stunt viroid* (HSVd) appeared to be absent from the SAM of infected hop plants. Shoot tips (0.2 mm) of infected plants bearing the apical dome and two youngest pairs of leaf primordia did not show abnormalities of cell wall structures visible in cells from the third primordium and below, no HSVd could be detected in the same 0.2-mm shoot tip by bioassay, and HSVd-free plants could be generated by meristem tip culture of the excised 0.2 to 0.3-mm shoot tips. Plants generated from larger shoot tips contained HSVd. Meristem culture experiments also suggest that PSTVd does not invade the shoot apex (Stace-Smith and Mellor, 1970; Lizárraga *et al.,* 1980). Our examination of infected tomato and *N. benthamiana* plants by *in situ* hybridization provides clear cytological evidence for the inability of PSTVd to invade the SAM.

Although we cannot formally rule out the possibility that absence of PSTVd in the SAM is partially or completely due to its failure to replicate in the SAM, we favor the hypothesis that plasmodesmata at some cellular boundaries between the SAM and the rest of plant body restrict PSTVd trafficking into the SAM. This is reminiscent of the situation in systemically acquired gene si-

lencing, where a nucleotide sequence-specific signal generated ectopically in the lower part of a plant is able to trigger gene silencing in the upper part of the plant, with notable exception of the shoot apex and mature leaves (Voinnet *et al.,* 1998). Data presented by these authors suggest that the signal fails to traffic into the shoot apex. Further studies are required to determine whether the trafficking of PSTVd and gene silencing signals is controlled by a common set of mechanisms at the molecular level.

The presence of PSTVd in the sepals but not in the ovary, petals, or stamens of infected plants was unexpected, given that vascular connections exist in all floral parts. Furthermore, the ovary, stamens, and petals are strong sinks that depend entirely on import of nutrients (and perhaps certain signals) via the phloem to sustain growth and development. At least three possible explanations could account for our observations. First, PSTVd may be transported in the sieve tubes but fail to enter companion/phloem parenchyma cells to replicate and invade subsequently neighboring nonvascular cells in the ovary, stamens, and petals. This scenario would suggest the presence of a mechanism to distinguish and selectively ferry RNAs (and perhaps also proteins) from a sieve element into its associated companion and phloem parenchyma cells. Second, despite functional continuity of phloem connections between all tissues and organs in the vegetative and reproductive shoot apices (Imlau *et al.,* 1999), transport within a sieve tube per se may be selective for certain macromolecules, especially at a point where the vascular system branches into specific tissues/organs. For instance, at the vascular junction between the ovary, stamens, petals, and sepals, incoming transport cargoes in the sieve tubes may be sorted and delivered to appropriate floral parts depending on the physiological function of a cargo. In this case, transport within a sieve tube would be less like a simple free-flowing river (Sjölund, 1997) and more like well-controlled traffic in a canal that permits free passage of certain cargoes (e.g., photoassimilates and other small organic compounds) and selective passage of others (e.g., some proteins, RNAs, and pathogens). Third, PSTVd may indeed be transported into all floral parts but fail to replicate in the ovary, petals, and stamens due to lack of a host factor(s) required to support replication or due to selective suppression by gene silencing. Such a gene silencing mechanism, if it exists, would be unique, because sequence-specific gene-silencing signals appear not to traffic into floral or vegetative apices (Voinnet *et al.,* 1998).

PSTVd has been reported to be seed transmitted in tomato (Benson and Singh, 1964; Singh, 1970). Seed transmission requires that the viroid infect the ovules and/or pollen; thus, it is possible that current *in situ* hybridization protocols are simply not sensitive enough to detect very low levels of PSTVd being transported into these tissues. Alternatively, particular growth or environmental condition may render these floral parts susceptible to PSTVd invasion. More extensive analyses (e.g., *in situ* PCR) are clearly necessary to resolve this issue.

In the absence of a mechanism to sustain its population during trafficking from the initial infection site to remote cells via the phloem, PSTVd concentrations might fall below those required to initiate new infections. Two alternative, though not mutually exclusive, mechanisms could sustain such infectious PSTVd populations: (i) PSTVd replicates actively in the phloem during long-distance movement, or (ii) replication of PSTVd in nonvascular cells at an initial infection site constantly supplies PSTVd to the phloem for long-distance transport. Our data indicate that PSTVd replicates in the phloem. As shown in Fig. 5, at least some PSTVd molecules may exit the sieve elements during transport and enter the phloem parenchyma and companion cells for replication, thereby generating more PSTVd molecules that then reenter sieve elements for further long-distance trafficking. Thus, replication along the transport pathway functions to propagate the PSTVd population for further trafficking as well as to provide infectious PSTVd molecules to invade neighboring nonvascular cells. We suggest that a similar strategy could be used by some regulatory RNAs to amplify and traffic systemically in a plant. Indeed, such a strategy could explain well the observed long-distance relay of ectopically and locally produced gene silencing signals (Voinnet *et al.,* 1998). Tobacco mosaic virus, which encodes a movement protein for cell-to-cell movement, may also require replication in companion and phloem cells to sustain long-distance movement during systemic infection of grafted tobacco plants (Arce-Johnson *et al.,* 1997).

In conclusion, our analyses suggest that PSTVd utilizes cellular mechanisms for systemic trafficking. Further characterization of viroid trafficking should yield valuable information on basic plant functions such as phloem transport, cell-to-cell transport, endogenous RNA trafficking, and gene regulation in addition to pathogen movement.

MATERIALS AND METHODS

Plant material

Tomato (*Lycopersicum esculentum* cv. Rutgers) seeds were planted in flats and allowed to germinate in a growth chamber maintained at 14-h day (30°C)/10-h night (20°C) temperature regime. When 3–5 cm tall, individual seedlings were transplanted into 10 × 10 cm containers holding standard soil mix (American Plant Products & Services, Inc., Oklahoma City, OK). *Nicotiana benthamiana* were planted and maintained under similar conditions, except that the day/night temperatures were 24°C/20°C.

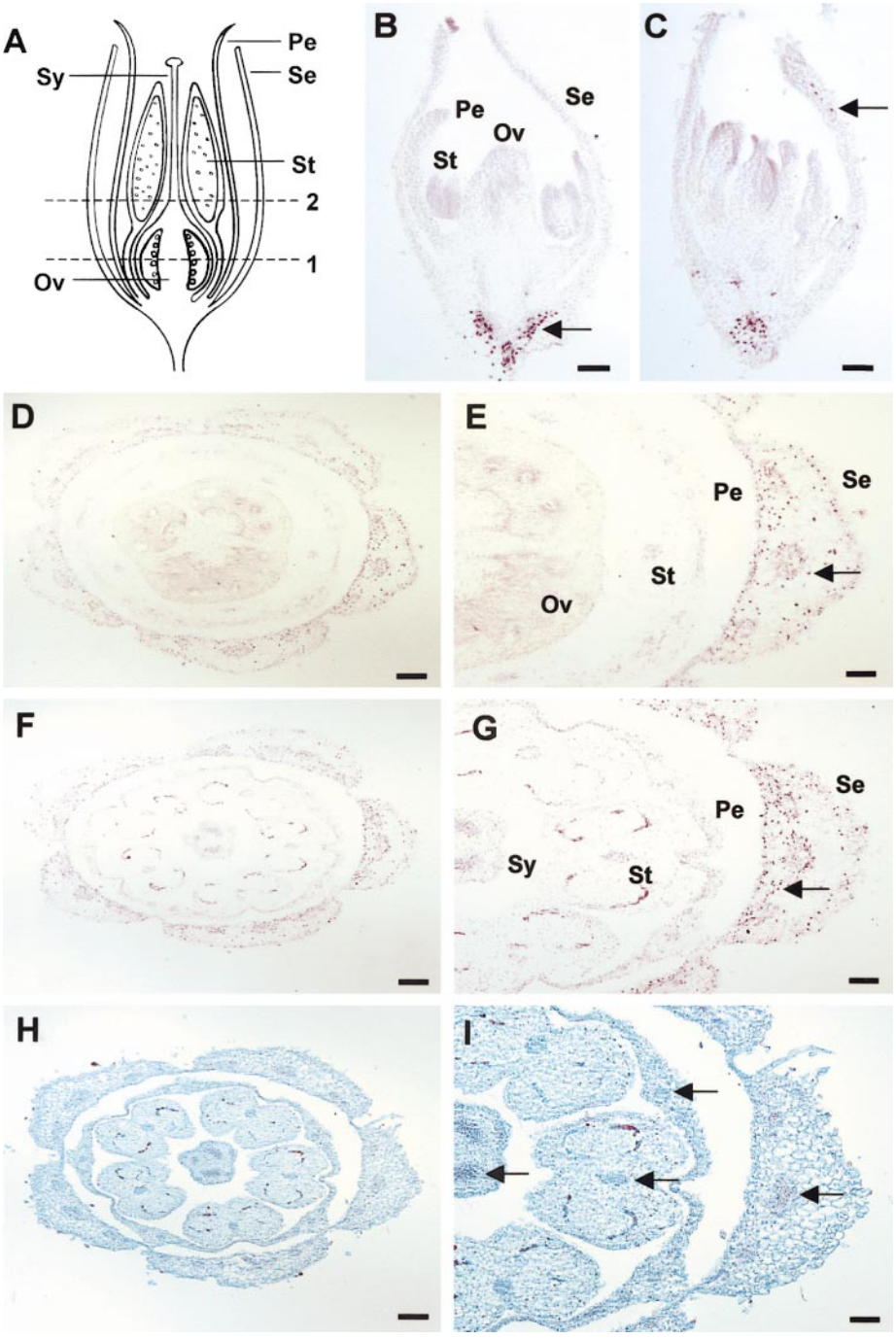

FIG. 3. PSTVd in flowers of infected *N. benthamiana* and tomato. (A) Longitudinal view of a tomato flower, showing various floral parts. Dashed line 1 indicates transverse section plane for images in (D) and (E), and dashed line 2 indicates transverse section plane for images in (F–I). Se, sepal; Pe, petal; St, stamen; Ov, ovary. (Adapted from Esau, 1965.) (B, C) Serial longitudinal sections of a developing *N. benthamiana* flower. Note the presence of PSTVd in the vascular tissue in the sepal (Se) and in the receptacle below the floral parts (arrows). The viroid is absent in petals (Pe), stamens (St), and the ovary (Ov). Bar = 30 μm. (D, E) Transverse section of a more developed tomato flower, obtained at position indicated by dashed line 1 in (A). PSTVd (arrow) is present in all parenchyma cells in the sepal (Se). The viroid is absent in petals (Pe), stamens (St), and the ovary (Ov). The right half of image (D) is magnified in (E). Bar in (D) = 80 μm. Bar in (E) = 40 μm. (F, G) Transverse section of the same flower as in (D) and (E), obtained at position indicated by dashed line 2 in (A). PSTVd (arrow) is present in the sepals (Se). The viroid is absent in petals (Pe), stamens (St), and the style (Sy). Bar in (F) = 80 μm. Bar in (G) = 40 μm. (H, I) Transverse section from the same tomato flower as above, obtained near position indicated by dashed line 2 in (A). The section was stained with safranin and fast green to show vascular tissues (arrows) in sepals, petals, stamens, and the style. Bar in (H) = 80 μm. Bar in (I) = 40 μm.

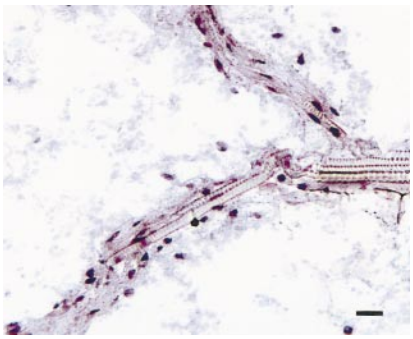

FIG. 4. PSTVd replication in the phloem. *In situ* hybridization of an infected tomato leaf detecting presence of the minus-sense, replicative intermediate form of PSTVd in companion and phloem parenchyma cells. Bar = 10 μm.

Preparation of PSTVd inocula

Plasmid pST64-B5, containing a full-length PSTVd (intermediate strain) cDNA (Owens *et al.,* 1986), was isolated from *E. coli* strain JM83 using the ClearCut Miniprep Kit from Stratagene (La Jolla, CA) following the manufacturer's instructions. For production of plus-strand PSTVd RNA transcripts, pST64-B5 DNA was linearized with *Eco*RI and used as templates for SP6 RNA polymerase-driven *in vitro* transcription using the MEGAscript Kit (Ambion Inc., Austin, TX) following the manufacturer's instructions. For mechanical inoculation, PSTVd transcripts or plasmid pST64-B5 DNA was diluted in 20 mM Na phosphate (pH 7.0) buffer and rubbed on Carborundum-dusted cotyledons of tomato seedlings. The buffer was used as the inoculum in control experiments.

An *Agrobacterium tumefaciens* strain whose modified binary vector pGA64 harbors a full-length PSTVd (intermediate strain) cDNA flanked by ribozymes and under the control of the CaMV 35S promoter was constructed by Hu *et al.* (1997). *Agrobacterium*-mediated inoculation followed the procedure of Hammond (1994). Briefly, approximately 100 μL of *A. tumefaciens* culture was placed on the stem of a 3-week-old tomato just below the oldest leaf. A sterile needle was used to stab the stem several times through the bacterial culture to a depth of approximately 1 mm. The plant was then returned to the growth chamber and allowed to continue growing. The presence or absence of PSTVd in leaflets collected from the uppermost leaf of each plant was determined by dot-blot hybridization (see below).

Hybridization probes

Digoxygenin (DIG) -labeled minus-strand PSTVd probes were prepared by *in vitro* transcription using as template *Eco*RI-linearized plasmid pST65-B5 (Owens *et al.,* 1986). DIG-labeled plus-strand PSTVd was prepared by *in vitro* transcription using as template *Eco*RI-linearized plasmid pST64-B5. DIG-UTP was purchased from Boehringer Mannheim (Indianapolis, IL) and *in vitro* transcription was carried out using the Ambion MEGAscript Kit (see above).

Dot-blot hybridization

Two to three weeks after inoculation, leaflets collected from the inoculated plants were assayed for the presence of PSTVd by dot-blot hybridization using a full-length, DIG-labeled minus-sense RNA probe. Leaf extracts were prepared by grinding 50–100 mg of leaf tissue in 100 μL of AMES buffer (0.5 M Na acetate of pH 7.5, 10 mM $MgCl_2$, 20% ethanol, 3% SDS, and 1 M NaCl; Laulhere and Rozier, 1976). The resulting homogenates were extracted with an equal volume of chloroform, and the clarified aqueous phase was transferred to a fresh microcentrifuge tube after centrifugation. Aliquots (2 μL) were spotted onto nylon membranes, and the membranes were irradiated with 1.2×10^5 μJ of UV light in a Stratalinker UV cross-linker (Stratagene).

Prehybridization was carried out at 55°C for 1 h with a solution containing 50% formamide, 5% dextran sulfate, 1% blocking reagent (Boehringer Mannheim), 0.3 M NaCl, 1 mM EDTA, and 150 μg/mL yeast tRNA (pH 7.5). After prehybridization, fresh solution containing 300 ng/mL DIG-labeled RNA probe was added and the membranes were incubated at 55°C for 19–20 h. After two washes with 2× SSC at 55°C (15 min each) and incubation (20 min at room temperature) in 2× SSC containing 2 μg/mL RNaseA, the membranes were washed twice (10 min each) with 0.1× SSC containing 0.1% SDS at 65°C and once (3 min) with maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5). Next, the membranes were blocked for 30 min in maleate buffer containing 1% blocking reagent (Boehringer Mannheim) and incubated for 30

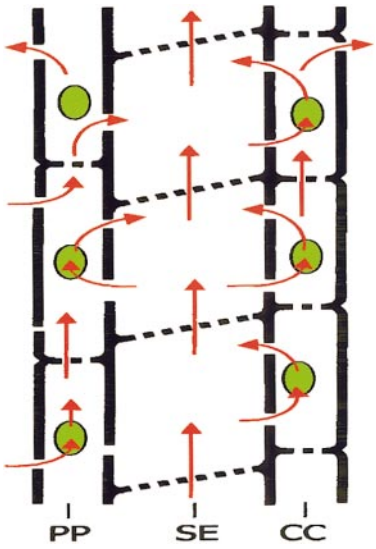

FIG. 5. A model for replication-supported long-distance trafficking of PSTVd. Orange arrows indicate directions of PSTVd movement. See text for details.

min with alkaline phosphatase-conjugated anti-DIG antibodies (Boehringer Mannheim; 1:5000 dilution). After two washes (15 min each) in maleate buffer and one wash (10 min) in AP buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the membranes were incubated in color substrate solution (100 μL of NBT/BCIP in 5 mL of AP buffer; Boehringer Mannheim) in the dark. Once color development was appropriate, the membranes were washed with TE buffer and water (10 min each) and air-dried.

Tissue processing for *in situ* hybridization

Plant samples were fixed in FAA (10% formaldehyde, 50% ethanol, and 5% acetic acid) at 4°C overnight. After dehydration and infiltration, the samples were embedded in paraffin (Electron Microscopy Sciences, Fort Washington, PA). Sections (8–10 μm) were obtained with a rotary microtome (Model 820, American Optical Co., Buffalo, NY) and placed on drops of water on gelatin-coated slides. After 5–10 min at room temperature, sections became flat, and excess water was removed from the edges using an absorbent tissue. Twenty to 30 min later, when the sections had dried completely, the slides were placed on a warming plate at 42–45°C overnight. This protocol significantly improved adherence of the sections to the slides during subsequent hybridization procedures. Slides were either used immediately for *in situ* hybridization or stored at 4°C until use.

In situ hybridization

The procedure used for *in situ* hybridization was adapted from several existing protocols (Jackson, 1991; Deblock and Debrouwer, 1993; Drews and Okamuro, 1996). Sections were dewaxed in xylene (2 changes, 10 min each) with gentle shaking and washed in 100% ethanol (2 changes, 5 min each). After hydration through a graded ethanol series (95, 85, 50, 30%) followed by sterile distilled water, the sections were prehybridized in blocking solution and then incubated with DIG-labeled PSTVd probes at 42°C overnight as describe above. Afterward, the sections were washed twice in 0.2× SSC at room temperature (30 min/change) and once at 55°C for 1 h. Next, the sections were incubated with alkaline phosphatase-conjugated anti-DIG antibodies (1:1250 dilution in a buffer of 100 mM Tris of pH 7.5, 150 mM NaCl, 0.3% Triton X-100, and 1% bovine serum albumin) for 2 h at room temperature. Following a final wash with AP buffer, the sections were incubated with the color substrate solution (100 μL of NBT/BCIP in 5 mL of AP buffer) in the dark. When color had developed sufficiently, the sections were mounted and examined under a Nikon Optiphot-2 or Eclipse 600 microscope (Nikon Corp., Tokyo, Japan). Images were captured and processed with a SPOT 2 CCD camera and the associated software (Diagnostics Instruments, Inc., Sterling Heights, MI).

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