Movement of Potato Spindle Tuber Viroid Reveals Regulatory Points of Phloem-Mediated RNA Traffic[1]

Yali Zhu, Yijun Qi, Yan Xun, Robert Owens, and Biao Ding*

Department of Plant Biology and Plant Biotechnology Center, Ohio State University, Columbus, Ohio 43210 (Y.Z., Y.Q., Y.X., B.D.); and Molecular Plant Pathology Laboratory, Agricultural Research Services, United States Department of Agriculture, Beltsville, Maryland 20705 (R.O.)

Increasing evidence indicates that the phloem mediates traffic of selective RNAs within a plant. How an RNA enters, moves in, and exits the phloem is poorly understood. Potato spindle tuber viroid (PSTVd) is a pathogenic RNA that does not encode proteins and is not encapsidated, and yet it replicates autonomously and traffics systemically within an infected plant. The viroid RNA genome must interact directly with cellular factors to accomplish these functions and is, therefore, an excellent probe to study mechanisms that regulate RNA traffic. Our analyses of PSTVd traffic in *Nicotiana benthamiana* yielded evidence that PSTVd movement within sieve tubes does not simply follow mass flow from source to sink organs. Rather, this RNA is transported into selective sink organs. Furthermore, two PSTVd mutants can enter the phloem to spread systemically but cannot exit the phloem in systemic leaves of tobacco (*Nicotiana tabacum*). A viroid most likely has evolved structural motifs that mimic endogenous plant RNA motifs so that they are recognized by cellular factors for traffic. Thus, analysis of PSTVd traffic functions may provide insights about endogenous mechanisms that control phloem entry, transport, and exit of RNAs.

RNA and protein traffic is a biological function basic to all multicellular organisms. Increasing evidence indicates that selective plant RNAs traffic from cell to cell through plasmodesmata and even from organ to organ through the phloem. Examples include Suc transporter 1 (*SUT1*) mRNA (Kühn et al., 1997), over 100 mRNAs from phloem exudates of pumpkin (Ruiz-Medrano et al., 1999), and a mutant fusion transcript between *LeT6*, a tomato (*Lycopersicon esculentum*) homeodomain protein gene, and *PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE* gene (Kim et al., 2001). Gene silencing signals, believed to consist of RNAs, also traffic systemically (Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Voinnet et al., 1998). These findings raise the prospect that systemic RNA traffic controls various plant developmental and physiological processes, in addition to surveillance and elimination of viral pathogens (Jorgensen et al., 1998; Lucas et al., 2001; Ueki and Citovsky, 2001; Wu et al., 2002).

The diversity of RNAs transported in the phloem poses questions about how the phloem delivers all cargos to the proper locations. Most important of these are whether a transport cargo has a distinct motif for traffic, whether entering and exiting the phloem involve the same or unique RNA motifs, and what cellular factors recognize and transport an RNA to its final destination (Oparka and Turgeon, 1999; Citovsky and Zambryski, 2000; Lucas et al., 2001; Ueki and Citovsky, 2001). Viroid infection provides a unique experimental system to study phloem-mediated RNA traffic in plants. Viroids are single-stranded, covalently closed circular, and pathogenic RNAs that infect plants (Riesner and Gross, 1985; Flores et al., 1997; Diener, 2001). Although they do not encode proteins, viroids can replicate autonomously and traffic systemically throughout their host plants. A viroid genome must apparently interact directly with host components for traffic.

We have been using potato spindle tuber viroid (PSTVd) as a model system to study RNA traffic in plants. This viroid replicates in the nucleus. Its systemic movement, therefore, includes nuclear transport (Woo et al., 1999; Zhao et al., 2001), cell-to-cell transport (Ding et al., 1997), and phloem transport (Palukaitis, 1987; Zhu et al., 2001). In this study, we used several approaches to obtain evidence that (a) a phloem-based mechanism transports PSTVd to sepals but not to the other floral organs of an infected plant, (b) PSTVd replication and phloem exit are mediated by different viroid motifs, and (c) phloem exit of PSTVd is potentiated by specific interactions with host factors. We present our results and discuss their biological implications.

RESULTS

PSTVd Trafficked into Selective Floral Organs

Our previous work showed that PSTVd intermediate ($PSTVd^{Int}$) strain was present in the sepals but not

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* Corresponding author; e-mail ding.35@osu.edu; fax 614–292–5379.

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in the petals, stamens, and ovary of developing flowers of mechanically inoculated tomato and *Nicotiana benthamiana* (Zhu et al., 2001). We analyzed further the PSTVd infection pattern in mature flowers of mechanically inoculated *N. benthamiana* by in situ hybridization. As shown in Figure 1A, the viroid was detected in sepals but not in the other floral organs. Thus, PSTVd consistently infected sepals, but not petals, stamens, and ovary of flowers at different stages. Considering that petals, stamens, and ovary are complete sink organs with functional phloem connections to the rest of the plant body and that *CmNACP*, *CmGAIP*, and *CmPP16* mRNAs (Ruiz-Medrano et al., 1999) and the green fluorescent protein (GFP; Imlau et al., 1999) can traffic into all floral organs, two possibilities could account for the presence of PSTVd in sepals and its absence in the other floral organs. First, PSTVd was transported into all floral organs, but its replication in the ovary, petals, and stamens was inhibited. Second, PSTVd was transported into sepals but not into the other floral organs.

To distinguish between these possibilities, we tested the ability of PSTVd to replicate in various floral organs of transgenic *N. benthamiana* that expresses the cDNA of PSTVd^{Int} under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Hu et al., 1997). We carried out in situ hybridization using a digoxigenin (DIG)-labeled RNA probe specific for the (−)-strand PSTVd, which is produced only during PSTVd replication (Branch and Robertson, 1984). Examination of flowers at various developmental stages from the transgenic plants revealed presence of the (−)-strand PSTVd in sepals, petals, stamens, and ovary (Fig. 1B). Furthermore, sequence analysis of PSTVd progeny isolated from the transgenic plants revealed wild-type (intermediate) sequence. We emphasize that the hybridization signal in the transgenic plants must be from PSTVd that accumulated as a result of autonomous viroid replication by using the CaMV 35S promoter-generated primary transcripts as the initial templates and not because of aberrant expression of the inserted PSTVd cDNA. The CaMV 35S promoter-generated primary transcripts are not detectable by RNA-RNA hybridization (Wassenegger et al., 1994; Hu et al., 1997), presumably because the viroid RNA-RNA replication leads to methylation of the viroid cDNA and, therefore, inactivation of further transcription (Wassenegger et al., 1994). As shown in Figure 1C, no hybridization signal was detected from transgenic *N. benthamiana* expressing only the central conserved region (Hu et al., 1997).

Our data indicate that PSTVd can replicate in all floral organs in *N. benthamiana.* Therefore, absence of PSTVd signals in petals, ovary, and stamens in mechanically inoculated plants can be best interpreted as being attributable to restricted traffic of PSTVd into these organs and not to suppression of replication.

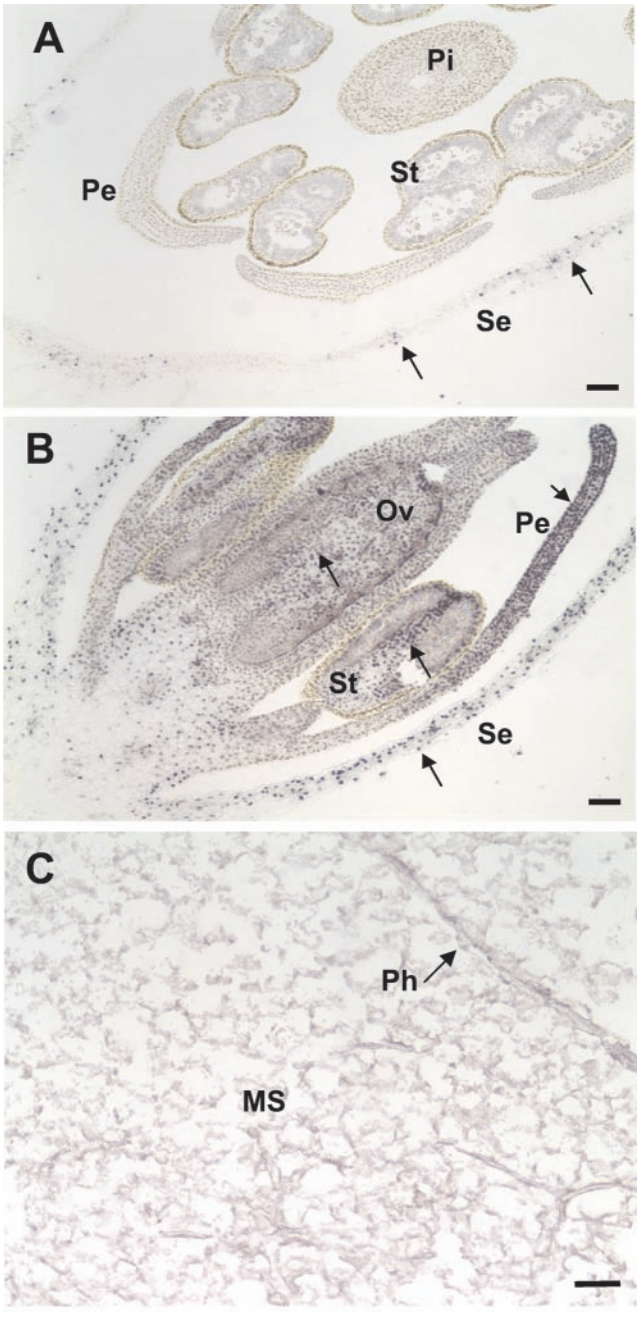

Figure 1. Selective traffic of PSTVd^{Int} into sepals but not into other floral organs of *N. benthamiana*, as detected by in situ hybridization on paraffin sections. A, Transverse view of a mature flower from mechanically inoculated *N. benthamiana*. The viroid signal (arrows) is detected in sepals (Se) but not in petals (Pe), stamens (St), or the pistil (Pi). B, Longitudinal view of a mature flower from a 35S:PSTVd^{Int}-transgenic *N. benthamiana* plant. Viroid signal is detected in all floral organs (arrows). Ov, Ovary. C, Absence of hybridization signal in leaf cells of transgenic *N. benthamiana* expressing the central conserved region of PSTVd under the control of the CaMV 35S promoter. MS, Mesophyll; Ph, phloem. All bars = 40 μm.

Two PSTVd Mutants Replicated in But Did Not Exit the Phloem in Tobacco (*Nicotiana tabacum*)

After mechanical inoculation, PSTVd^{Int} strain infects *N. benthamiana* and tomato systemically (Zhu et al., 2001), but it hardly infects tobacco (*N. tabacum*). The lack of infection in tobacco could be attributable to inability of the viroid to replicate in tobacco cells and/or to move systemically. Tobacco infection would, therefore, provide a complementary experimental system to gain further insights about viroid-host interactions for systemic traffic.

We first tested whether PSTVd would replicate in the phloem of tobacco and then exit the phloem to invade nonvascular tissues. To overcome the technical barrier of mechanically inoculating the phloem, we generated transgenic plants expressing the cDNA of PSTVd^{Int} under the control of the companion cell-specific commelina yellow mottle virus (CoYMV) promoter (Matsuda et al., 2002; Fig. 2A). Previous studies on transgenic tobacco and *N. benthamiana* with CaMV 35S promoter-driven expression of PSTVd cDNAs have established the validity of this inoculation approach. As discussed above, the primary transcripts of PSTVd derived from the promoter activity would serve as the templates to initiate autonomous RNA-RNA replication of the viroid (Wassenegger et al., 1994; Hu et al., 1997).

We obtained two CoYMV:PSTVd^{Int}-transgenic tobacco lines (4 and 8) that showed viroid accumulation in leaves based on dot-blot analysis (data not shown). We then performed in situ hybridization to determine the cellular localization of the viroid, using DIG-labeled RNA probes specific for the (−)-strand of PSTVd. We have shown previously that PSTVd traffics from the phloem into all other cells in young sink leaves but is restricted to the phloem during sink-to-source transition of a leaf in *N. benthamiana* and tomato as a result of changes in leaf physiology during development (Zhu et al., 2001). Therefore, in this and subsequent experiments, we focused on analysis of PSTVd traffic in sink leaves. Our analyses showed that the viroid signal was present only in the phloem of tobacco (Fig. 2, B–E). In contrast, the viroid was detected in the phloem, mesophyll, and epidermal cells of CoYMV:PSTVd^{Int}-transgenic *N. benthamiana* (Fig. 2, F and G). These data indicate that in an appropriate host, companion cell-derived viroid can traffic into surrounding cells for replication. In transgenic tobacco, lack of interactions with a positive host factor or active interactions with a negative host factor may have contributed to the restriction of PSTVd in the phloem.

We used reverse transcription (RT)-PCR) to amplify viroid progeny from all transgenic lines for sequencing. We also used PCR to amplify the PSTVd cDNA inserted into the genomes of these plant lines for sequencing. The viroid progeny from the transgenic *N. benthamiana* maintained the Intermediate sequence. Surprisingly, PSTVd progeny in line 4 of transgenic tobacco contain $C_{259} \rightarrow U$ change and in line 8 contain $U_{257} \rightarrow A$ change. The viroid cDNA sequence inserted into the plant genome was not altered in any lines.

The $C_{259} \rightarrow U$ change is identical to the mutation that converts the tomato-strain PSTVd KF440–2 into the tobacco-infectious strain PSTVd-NT (Wassenegger et al., 1996). We designate our two mutants as PSTVd^{Int} $U_{257} \rightarrow A$ and PSTVd^{Int} $C_{259} \rightarrow U$, respectively. We should point out that PSTVd^{Int} $C_{259} \rightarrow U$ and PSTVd-NT, although both derived from $C_{259} \rightarrow U$ substitution, are not identical because their respective parental strains PSTVd^{Int} and PSTVd KF440–2 have five nucleotide differences in the pathogenicity domain (Schnölzer et al., 1985).

To test further whether PSTVd^{Int} $U_{257} \rightarrow A$ and PSTVd^{Int} $C_{259} \rightarrow U$ were confined to the phloem in infected tobacco, we constructed cDNAs of these mutants to carry out two types of experiments. First, we mechanically inoculated in vitro transcripts derived from these cDNAs onto tobacco. In situ hybridization on young leaves of positively infected plants revealed presence of the viroid exclusively in the phloem (Fig. 2H). The viroid progeny from the infected plants were sequenced to confirm maintenance of the mutant sequences, respectively. Second, we generated transgenic tobacco expressing the cDNAs of these mutants under the control of the CoYMV promoter, respectively. In situ hybridization again showed that the viroid was confined to the phloem (data not shown), and sequence analysis also confirmed maintenance of the mutant sequences. We then tested whether $U_{257} \rightarrow A$ or $C_{259} \rightarrow U$ mutation would impair replication and/or traffic of PSTVd in *N. benthamiana*. We mechanically inoculated in vitro transcripts of both PSTVd mutants onto this plant. In situ hybridization showed that both PSTVd^{Int} $U_{257} \rightarrow A$ (Fig. 2I) and PSTVd^{Int} $C_{259} \rightarrow U$ (data not shown) infected all cells of systemic leaves. The mutant sequences were maintained in the infected plants. Thus, both $U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ mutations did not abolish PSTVd replication or systemic movement in *N. benthamiana*. Altogether, these data provided compelling evidence that PSTVd^{Int} $U_{257} \rightarrow A$ and PSTVd^{Int} $C_{259} \rightarrow U$ replicated in and were confined to the phloem in tobacco. As shown below, this confinement was because of the inability of these mutants to exit the phloem.

PSTVd^{Int} $C_{259} \rightarrow U$ and PSTVd^{Int} $U_{257} \rightarrow A$ Replicated in Nonvascular Tissues of Tobacco

Presence of PSTVd^{Int} $U_{257} \rightarrow A$ and PSTVd^{Int} $C_{259} \rightarrow U$ in the phloem and its absence from all other cells in transgenic tobacco raised a number of questions. First, was the phloem restriction attributable to inability of the viroid to traffic out of the phloem into nonvascular tissues such as the mesophyll for replication? Second, did the viroid traffic into nonvascu-

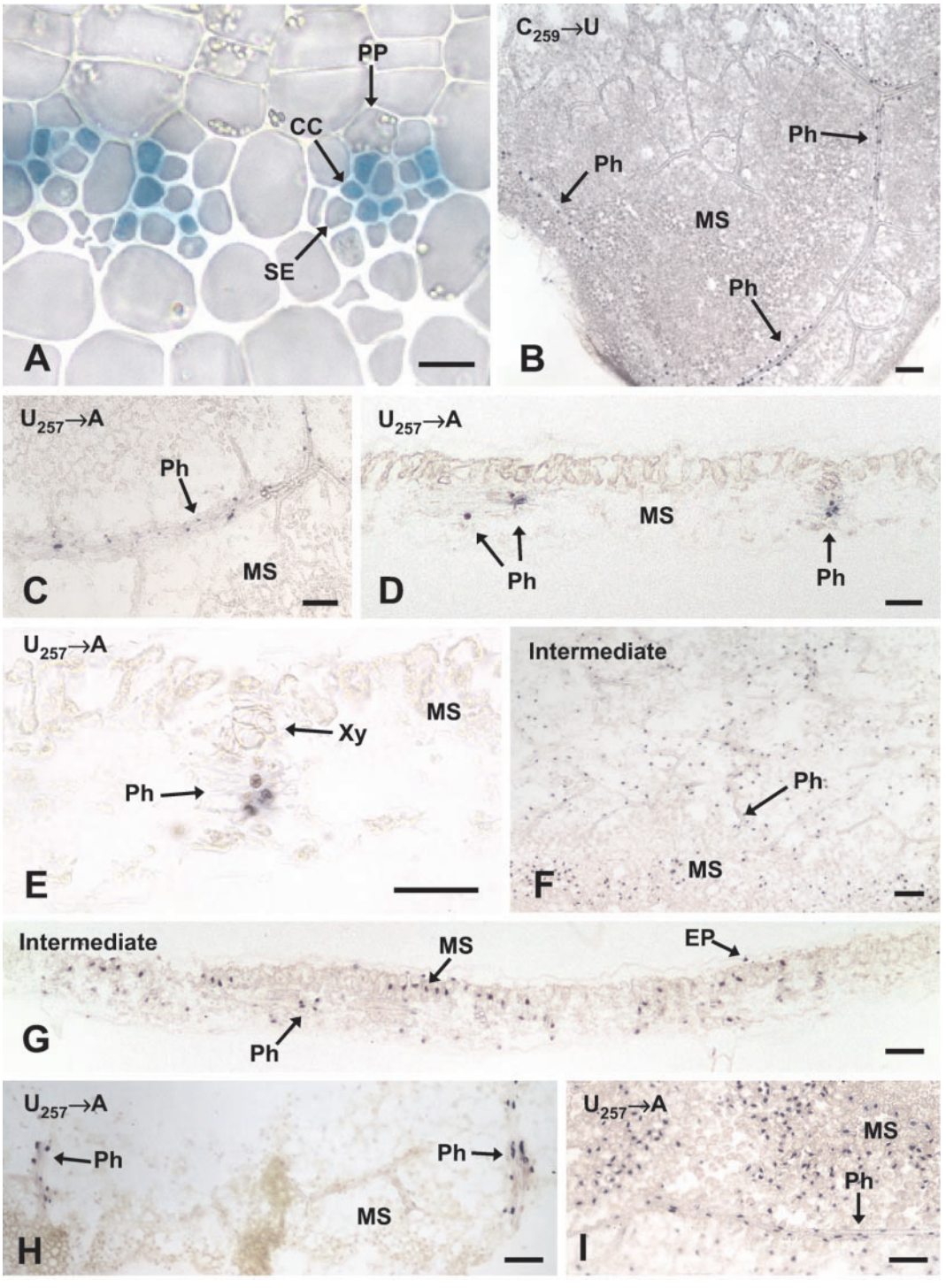

Figure 2. Restricted traffic of replicating PSTVd out of the phloem in CoYMV:PSTVd-transgenic tobacco but not in transgenic *N. benthamiana*. PSTVd is detected in the nuclei (purple dots in B–I) by in situ hybridization; H is from a cryosection, and all others are from paraffin sections. A, Companion cell (CC)-specific activity of the CoYMV promoter in transgenic tobacco, as revealed by β-glucuronidase (GUS) reporter expression (Matsuda et al., 2002). The GUS activity is absent from all other cells including sieve element (SE) and phloem parenchyma (PP). B through E, CoYMV:PSTVd^{Int}-transgenic tobacco. Paradermal (B and C) and transverse (D and E) leaf sections show that PSTVd mutants replicate and remain in the phloem (Ph). The phloem tissue on the left of D is obliquely sectioned. E, A high magnification view of the right portion of D. MS, Mesophyll; Xy, xylem. F and G, CoYMV:PSTVd^{Int}-transgenic *N. benthamiana*. Paradermal (F) and transverse (G) leaf sections show that PSTVd^{Int} accumulates in phloem (Ph), mesophyll (MS), and epidermis (EP). H, Presence of PSTVd^{Int} $U_{257} \rightarrow A$ in the phloem (Ph) of a systemic leaf of mechanically inoculated tobacco. MS, Mesophyll. I, Presence of PSTVd^{Int} $U_{257} \rightarrow A$ in the phloem (Ph) and mesophyll (MS) of a systemic leaf of mechanically inoculated *N. benthamiana*. Bar in A = 10 μm. Bars in B through I = 40 μm.

lar tissues but not replicate in these tissues? Third, are $U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ changes essential for PSTVd replication in tobacco?

To address these questions, we generated transgenic tobacco expressing the cDNA of PSTVd^{Int} under the control of the CaMV 35S promoter. Ten transgenic lines were obtained that showed accumulation of PSTVd based on dot-blot analysis (data not shown). To determine the cellular localization of PSTVd in these transgenic plants, we performed in situ hybridization using a DIG-labeled RNA probe specific for the (−)-strand PSTVd. The viroid was detected in patches of mesophyll and phloem cells (Fig. 3, A and B), indicating that the viroid can replicate in these tissues. In transgenic *N. benthamiana* expressing the cDNA of the PSTVd^{Int} under the control of the CaMV 35S promoter, the viroid was also detected in all leaf cells (Hu et al., 1997; Fig. 3C).

Sequence analysis revealed that viroid progeny isolated from transgenic *N. benthamiana* maintained the PSTVd^{Int} sequence. In contrast, viroid progeny from seven of the transgenic tobacco lines contain $C_{259} \rightarrow U$ change and progeny from the other three tobacco lines contain $U_{257} \rightarrow A$ change. These changes are identical to those obtained from the CoYMV:PSTVd^{Int}-transgenic plants. The viroid cDNA sequences inserted into the plant genome in all transgenic plants were not altered.

Our data indicate that $U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ substitutions in PSTVd^{Int} are important for replication in tobacco, similar to the conversion of the tomato-strain PSTVd KF440–2 into the tobacco-infectious strain PSTVd-NT by the $C_{259} \rightarrow U$ substitution (Wassenegger et al., 1996). As suggested by Wassenegger et al. (1996), a very low level of PSTVd replication, using primary transcripts derived from the 35S promoter activity as the initial templates, may have occurred in transgenic tobacco. During this replication, mutations occurred randomly and $U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ changes enhanced some aspects of replication and accumulation. It is puzzling why the viroid appeared in patches of cells. Two explanations are possible. First, PSTVd^{Int} $U_{257} \rightarrow A$ or PSTVd^{Int} $C_{259} \rightarrow U$ mutations arose independently in a few cells of the mesophyll and phloem, and these variants replicate and move into surrounding cells (within the phloem and mesophyll, respectively). Second, PSTVd^{Int} $U_{257} \rightarrow A$ or PSTVd^{Int} $C_{259} \rightarrow U$ arose in a few cells in the early stages of organogenesis. Subsequent cell division spread the viroid population into multiple and contiguous cells. These potential mechanisms are not necessarily mutually exclusive. They could have all contributed to the observed accumulation of PSTVd^{Int} $U_{257} \rightarrow A$ or PSTVd^{Int} $C_{259} \rightarrow U$ in patches of cells in the transgenic plants.

Because PSTVd^{Int} $U_{257} \rightarrow A$ and PSTVd^{Int} $C_{259} \rightarrow U$ can replicate in tobacco mesophyll and in the phloem, we attribute their phloem limitation in the CoYMV:PSTVd^{Int}-transgenic tobacco and in the me-

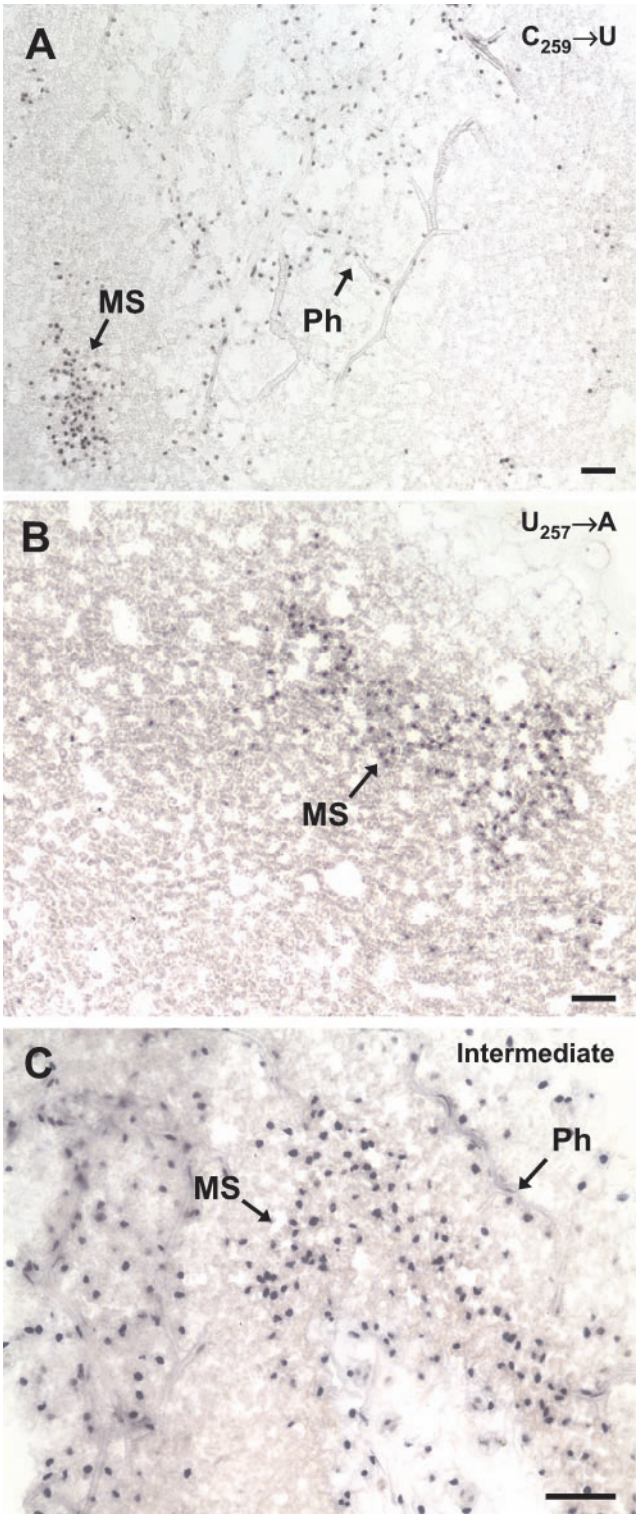

Figure 3. Replication of PSTVd in mesophyll of transgenic tobacco and *N. benthamiana*. As detected by in situ hybridization on paraffin sections, each purple dot represents localization of PSTVd in the nucleus. A and B, Accumulation of PSTVd^{Int} $C_{259} \rightarrow U$ and PSTVd^{Int} $U_{257} \rightarrow A$ in mesophyll (MS) and phloem (Ph) of CaMV 35S:PSTVd^{Int}-transgenic tobacco, respectively. C, Accumulation of PSTVd^{Int} in mesophyll (MS) and phloem (Ph) of CaMV 35S:PSTVd^{Int}-transgenic *N. benthamiana*. Bars = 40 μm.

chanically inoculated tobacco to their inability to traffic out of the phloem. Our data, thus, suggest that PSTVd replication and phloem-to-nonvascular tissue traffic are mediated by separate viroid motifs.

$U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ Substitutions Did Not Alter PSTVd Structure

Because PSTVd does not encode proteins, all functional information resides directly within the RNA genome itself. To gain insights into the molecular basis of $U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ substitutions for the enhanced replication in tobacco, we analyzed the structure of $PSTVd^{Int}$ $U_{257} \rightarrow A$ and $PSTVd^{Int}$ $C_{259} \rightarrow U$ in comparison with that of the $PSTVd^{Int}$. Computing with mfold (version 3.1 for 37°C; Zuker et al., 1999) or RNAstructure 3.6 (Mathews et al., 1999) revealed no difference in the secondary structure of the three strains (Fig. 4). Data suggest that nucleotide changes, and not structural changes, led to the enhanced replication of PSTVd in tobacco. However, alternative approaches (e.g. chemical/enzymatic mapping and x-ray crystallography) are needed to verify independently the computed secondary structure and/or to uncover potential tertiary structures.

DISCUSSION

It is generally believed that, once in the phloem, long-distance movement of macromolecules including viral pathogens follows mass flow of photoassimilates from source to sink organs/tissues (Leisner and Turgeon, 1993; Roberts et al., 1997; Golecki et al., 1999). Recent studies showed that the 27-kD GFP ectopically produced in the companion cells of mature source leaves is loaded into sieve elements and transported to sink leaves in transgenic Arabidopsis and tobacco (Imlau et al., 1999; Oparka et al., 1999). Imlau et al. (1999) further showed that GFP can traffic all the way into shoot apices and all floral organs. These observations have led to speculations that macromolecular flow from sieve elements to neighboring cells in a sink organ occurs by default without regulation (Oparka and Santa Cruz, 2000; Zambryski and Crawford, 2000).

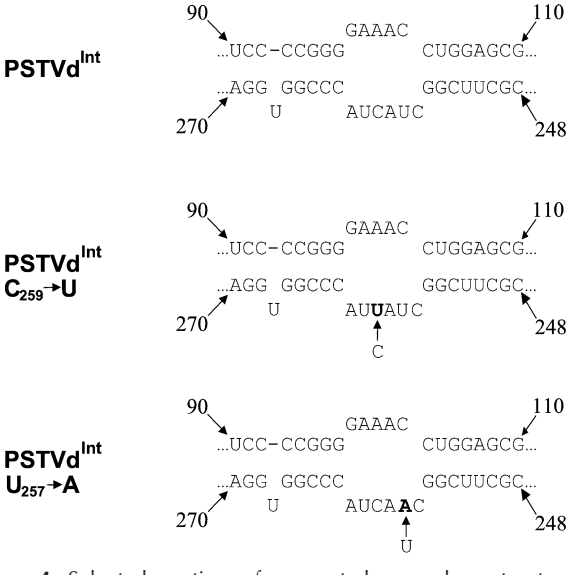

Figure 4. Selected portion of computed secondary structure of $PSTVd^{Int}$, $PSTVd^{Int}$ $C_{259} \rightarrow U$, and $PSTVd^{Int}$ $U_{257} \rightarrow A$. Nucleotide substitutions $C_{259} \rightarrow U$ and $U_{257} \rightarrow A$ are indicated.

Our data demonstrate that $PSTVd^{Int}$ can replicate in all floral organs of transgenic *N. benthamiana* plants, and yet the viroid is present only in sepals but not in petals, stamens, and ovary in mechanically inoculated *N. benthamiana*. Considering these observations and the finding that some plant mRNAs can traffic into all floral organs (Ruiz-Medrano et al., 1999), we suggest that a phloem-based mechanism selectively transports $PSTVd^{Int}$ into sepals but not into other floral organs. On the basis of this model, flow of macromolecules within sieve tubes to sink organs does not occur all by default. At present, we cannot exclude the possibility that the viroid is transported into the sieve elements of petals, stamens, and ovary but is not unloaded into surrounding nucleate cells for replication. In this case, the small amount of $PSTVd^{Int}$ in individual sieve elements could have escaped detection by the current in situ hybridization protocol. Whether traffic of PSTVd into various floral organs is regulated during passage within the sieve tubes or during exit from the sieve tubes, our observations support the hypothesis that the phloem has a sophisticated mechanism to deliver macromolecules to specific sink organs.

Our studies on PSTVd traffic in leaves of tobacco and *N. benthamiana* provided compelling evidence that phloem exit of an RNA is highly controlled. Although $PSTVd^{Int}$ $U_{257} \rightarrow A$ and $PSTVd^{Int}$ $C_{259} \rightarrow U$ can replicate in the phloem and mesophyll cells of tobacco leaves, they do not traffic from the phloem to mesophyll. In *N. benthamiana*, these two mutants and the parental strain $PSTVd^{Int}$ can exit the phloem to invade neighboring tissues. These results lend strong support to the hypothesis that PSTVd contains structural motifs for intercellular movement (Ding et al., 1997). Consistent with this hypothesis, in situ hybridization of tomato roots and stems infected by mutant PSTVd-R revealed restriction of the mutant replication in the vascular tissue (Hammond, 1994). PSTVd-R contains UU→AA substitutions at positions 177 and 178 and an additional G residue between positions 176 and 177 (Hammond and Owens, 1987). It should be noted, however, that the replication function of mutant PSTVd-R in nonvascular tissues of these organs was not tested (Hammond, 1994).

Interestingly, fluorescently labeled in vitro transcripts of $PSTVd^{Int}$ can move from cell to cell when injected into tobacco mesophyll (Ding et al., 1997). Furthermore, $PSTVd^{Int}$ $U_{257} \rightarrow A$ or $PSTVd^{Int}$ $C_{259} \rightarrow$

U that was mechanically inoculated onto tobacco leaves apparently moved from epidermis to mesophyll and then into the phloem to spread systemically, but they did not exit the phloem in the systemic leaves. These results suggest that the PSTVd genome contains multiple structural motifs for traffic between different cell or tissue types. In particular, phloem entry and exit appear to be mediated by different motifs. There are examples that phloem entry and exit of a virus also use different mechanisms. Ghoshroy et al. (1998) showed that nontoxic concentrations of cadmium inhibit systemic infection of turnip vein clearing virus, otherwise an infectious virus, in tobacco. Cadmium has no effect on viral replication, assembly and local movement, but it appears to inhibit virus exit from the vascular tissue (Ghoshroy et al., 1998).

Our data showing that PSTVd^{Int} $U_{257} \rightarrow A$ and PSTVd^{Int} $C_{259} \rightarrow U$ can replicate in the phloem and mesophyll but cannot traffic from the phloem to nonvascular tissues provide evidence that replication and phloem exit are mediated by different viroid motifs. Notably, both $U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ mutations occur in the lower one-half of the same loop within the central conserved region of PSTVd. The analysis of Wassenegger et al. (1996) specifically showed that nucleotide C_{259} is part of the PSTVd loop E sequence, which is conserved in eukaryotic 5S rRNA (Wimberly et al., 1993), 28S rRNA (Szewczak and Moore, 1995), and viroid RNAs (Branch et al., 1985; Gast et al., 1996). Wassenegger et al. (1996) postulated that C_{259}, as an extrahelical residue in loop E, is involved in interacting with host factors for efficient replication in tobacco. Our isolation of the tobacco-infectious strain PSTVd^{Int} $C_{259} \rightarrow U$ confirms the importance of $C_{259} \rightarrow U$ for PSTVd replication in tobacco. U_{257} is significantly also part of the loop E (see Wassenegger et al., 1996). That both $U_{257} \rightarrow A$ and $C_{259} \rightarrow U$ are critical for PSTVd replication but not for phloem exit in tobacco further underscores the involvement of loop E in viroid replication and suggests that the viroid motif(s) for phloem exit is located elsewhere. Work is in progress to identify this traffic motif(s). In search of cellular factors that are involved in phloem traffic of viroids, two recent studies demonstrate that phloem lectin PP2 from cucumber phloem exudate binds viroids and other RNAs (Gómez and Pallás, 2001; Owens et al., 2001). Whether PP2 has a role in viroid traffic is being investigated.

In conclusion, our analyses of PSTVd traffic reveal regulatory points for systemic RNA traffic that likely involve distinct interactions between RNA motifs and cellular factors. RNA movement within sieve tubes may not simply follow mass flow from source to sink. It appears that some control mechanisms can sort and direct an RNA to selective sink organs. In addition, phloem exit of an RNA in sink organs is also highly controlled. A viroid most likely has evolved structural motifs that mimic endogenous plant RNA motifs so that they are recognized by cellular factors for traffic. In this regard, a viroid can be considered an exogenous RNA that contains "endogenous" traffic motifs and can, therefore, be used to study the mechanisms that control phloem entry, transport, and exit of plant RNAs.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Tobacco (*Nicotiana tabacum* cv Samsun) and *Nicotiana benthamiana* were grown in a growth chamber maintained at 27°C/22°C day/night temperature regimes and 14-/10-h light/dark cycle.

Molecular Cloning and Generation of Transgenic Plants

Two types of DNA constructs were generated for plant transformation. First, the cDNA of PSTVd^{Int} flanked by ribozymes (Hu et al., 1997) was inserted into *Sma*I site, downstream of the CaMV 35S promoter, of plasmid pRTL2 (Carrington and Freed, 1990; Restrepo et al., 1990). The 35S:PSTVd^{Int} construct was then inserted into *Hin*dIII site of binary vector pGA482. Second, the cDNA of PSTVd^{Int} was inserted into *Sma*I site of binary vector pCOI (provided by Dr. Gary Thompson [University of Arkansas, Little Rock]), downstream of the companion cell-specific CoYMV promoter (Matsuda et al., 2002). After sequence verification, these constructs were used to transform *Agrobacterium tumefaciens* (LBA 4404).

A standard *A. tumefaciens*-mediated leaf-disc transformation method (Horsch et al., 1985) was used to generate transgenic tobacco and *N. benthamiana* plants. Dot-blot analysis with RNA probes specific for the (+)-strand PSTVd was used for initial screening for transgenic lines that accumulated PSTVd. Protocols for dot-blot analysis were described earlier (Zhu et al., 2001).

Sequencing of PSTVd cDNA and RNA Progeny

Genomic DNA was extracted from PSTVd transgenic plants using DNeasy Plant Mini Kit (Qiagen USA, Valencia, CA). PSTVd was amplified by PCR from genomic DNA, using primers PSTVd-AS (5′-CCTGAAGCGCTCCTC-CGAG-3′) and PSTVd-S (5′-GATCCCCGGGGAAACCTGG-3′) and *PfuTurbo* DNA polymerase (Stratagene, La Jolla, CA) and the buffer provided by the manufacturer. The PCR cycling profile (28 cycles) was 30 s at 95°C, 30 s at 55°C, and 45 s at 68°C with a final extension step at 68°C for 10 min. The PCR products were fractionated on 1.5% (w/v) agarose gel. The band of expected size was cut out of the gel and purified with Qiaquick Gel Extraction Kit (Qiagen USA). *Taq* DNA polymerase (Promega, Madison, WI) was used to create 3′-A overhangs to the purified PCR products, which were then cloned into pGEM-T vector (Promega). PSTVd cDNAs in the plasmids were sequenced in both directions using the ABI377 DNA sequencer (PerkinElmer Life Sciences, Boston) at the DNA Sequencing Facility at The Ohio State University.

To determine the sequences of RNA progeny, total RNA was isolated from the PSTVd-transgenic plants or mechanically inoculated plants using RNeasy Plant Mini Kit (Qiagen USA) following manufacturer's instructions. cDNAs of PSTVd RNA were synthesized by RT-PCR. The first cDNA strand was synthesized using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA) and primer PSTVd-AS following the protocols recommended by the manufacturer. To synthesize the second strand of cDNA, aliquots (1/10) of the RT reaction mixture were PCR-amplified with primers PSTVd-AS and PSTVd-S as described. The PCR products were purified, cloned, and sequenced as described.

Construction of cDNA Clones of PSTVd^{Int} $U_{257} \rightarrow A$ and PSTVd^{Int} $C_{259} \rightarrow U$ for in Vitro Transcription

To generate cDNA clones of the PSTVd mutants for in vitro transcription, the 294-bp *Eag*I-*Eco*47 III fragment (positions 145–359/1–79) of the mutants

were transferred to a double ribozyme expression cassette pRZ6–2 (Hu et al., 1997) by replacing the corresponding fragment of the PSTVd^{Int}. This resulted in plasmids pRZ:IntU_{257} → A and pRZ:IntC_{259} → U.

Synthesis of in Vitro Transcripts and Mechanical Inoculation

For production of in vitro transcripts of the PSTVd^{Int} and the two mutants, plasmids pRZ6–2 (Hu et al., 1997), pRZ:IntU_{257} → A, and pRZ:IntC_{259} → U were linearized with *Hin*dIII and used as template for in vitro transcription using the T7 MEGAscript Kit (Ambion, Austin, TX) following manufacturer's protocol. After in vitro transcription, the DNA template was removed by digestion with RNase-free DNase. The RNA transcripts were purified using RNeasy Plant Mini Kit (Qiagen USA) and diluted to a final concentration of 100 ng μL^{-1} in 20 mM sodium phosphate buffer (pH 7.0). Aliquots of 10 μL were rubbed onto each of carborundum-dusted young leaves of 2-week-old tobacco or *N. benthamiana* seedlings.

Tissue Processing

Samples from transgenic or mechanically inoculated plants were processed to obtain paraffin sections or cryosections for in situ hybridization. Protocols for tissue processing to obtain paraffin sections were essentially as described in Zhu et al. (2001). In brief, plant samples were fixed in formaldehyde-acetic acid (10% [v/v] formaldehyde, 50% [v/v] ethanol, and 5% [v/v] 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).

Cryosections were obtained as described in Itaya et al. (1998). In brief, samples were fixed in a mixture of 3.7% (w/v) paraformaldehyde, 0.1% (v/v) glutaraldehyde, 0.2% (w/v) picric acid, 50 mM potassium phosphate, and 5 mM EGTA for 2 to 3 h. The fixed samples were infiltrated sequentially with 3:7 (v/v), 5:5 (v/v), and 7:3 (v/v) embedding mixture (two parts of 20% [w/v] Suc and one part of O.C.T. compound; Ted Pella Inc., Redding, CA):potassium phosphate/EGTA buffer. Afterward the samples were infiltrated with pure O.C.T. compound, embedded, and frozen at −20°C. The frozen samples were sectioned to 12 μm thickness using a cryostat (HM500, Microm, Walldorf, Germany).

In Situ Hybridization

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

In situ hybridization was performed as described previously (Zhu et al., 2001), using reagents purchased from Boehringer Mannheim. In brief, cryosections or dewaxed paraffin sections were prehybridized in a blocking solution and then incubated with DIG-labeled PSTVd probes. Afterward, the sections were washed in 0.2× SSC and then incubated with alkaline phosphatase-conjugated anti-DIG antibodies. Finally, the sections were incubated in the color substrate (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) solution. When color had developed sufficiently, the sections were mounted and examined under an Eclipse 600 microscope (Nikon, Tokyo). Images were captured and processed with a SPOT 2 Slider CCD camera and the associated software (Diagnostics Instruments, Sterling Heights, MI).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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LITERATURE CITED

Branch AD, Robertson HD (1984) A replication cycle for viroids and other small infectious RNAs. Science **223:** 450–454

Branch AD, Benenfeld BJ, Robertson HD (1985) Ultraviolet light-induced crosslinking reveals a unique region of local tertiary structure in potato spindle tuber viroid and HeLa 5S RNA. Proc Natl Acad Sci USA **82:** 6590–6594

Carrington JC, Freed DD (1990) Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. J Virol **64:** 1590–1597

Citovsky V, Zambryski P (2000) Systemic transport of RNA in plants. Trends Plant Sci **5:** 52–54

Diener TO (2001) The viroid: biological oddity or evolutionary fossil? Adv Virus Res **57:** 137–184

Ding B, Kwon MO, Hammond R, Owens R (1997) Cell-to-cell movement of potato spindle tuber viroid. Plant J **12:** 931–936

Flores R, Di Serio F, Hernández C (1997) Viroids: the non-encoding genomes. Semin Virol **8:** 65–73

Gast F-U, Kempe D, Ludwig R, Spieker RL, Sänger HL (1996) Secondary structure probing of potato spindle tuber viroid (PSTVd) and sequence comparison with other small pathogenic RNA replicons provides evidence for central non-canonical base-pairs, large A-rich loops, and a terminal branch. J Mol Biol **262:** 652–670

Ghoshroy S, Freedman K, Lartey R, Citovsky V (1998) Inhibition of plant viral systemic infection by non-toxic concentrations of cadmium. Plant J **13:** 591–602

Golecki B, Schulz A, Thompson GA (1999) Translocation of structural P proteins in the phloem. Plant Cell **11:** 127–140

Gómez G, Pallás V (2001) Identification of an in vitro ribonucleoprotein complex between a viroid RNA and a phloem protein from cucumber plants. Mol Plant-Microbe Interact **14:** 910–913

Hammond RW (1994) *Agrobacterium*-mediated inoculation of PSTVd cDNAs onto tomato reveals the biological effect of apparently lethal mutations. Virology **201:** 36–45

Hammond RW, Owens RA (1987) Mutational analysis of potato spindle tuber viroid reveals complex relationships between structure and infectivity. Proc Natl Acad Sci USA **84:** 3967–3971

Horsch RB, Fry J, Hoffmann NL, Wallroth M, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231

Hu Y, Feldstein PA, Hammond J, Hammond RW, Bottino PJ, Owens RA (1997) Destabilization of potato spindle tuber viroid by mutations in the left terminal loop. J Gen Virol **78:** 1199–1206

Imlau A, Truernit E, Sauer N (1999) Cell-to-cell and long-distance trafficking of the green fluorescent protein in the phloem and symplastic unloading of the protein into sink transport. Plant Cell **11:** 309–322

Itaya A, Woo Y-M, Masuta C, Bao Y, Nelson R, Ding B (1998) Developmental regulation of intercellular protein trafficking through plasmodesmata in tobacco leaf epidermis. Plant Physiol **118:** 373–385

Jorgensen RA, Atkinson RG, Forster RLS, Lucas WJ (1998) An RNA-based information superhighway in plants. Science **279:** 1486–1487

Kim M, Canio W, Kessler S, Sinha N (2001) Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. Science **293:** 287–289

Kühn C, Franceschi VR, Schulz A, Lemoine R, Frommer WB (1997) Macromolecular trafficking indicated by localization and turnover of sucrose transporters in enucleate sieve elements. Science **275:** 1298–1300

Leisner SM, Turgeon R (1993) Movement of virus and photoassimilate in the phloem: a comparative analysis. Bioassays **15:** 741–748

Lucas WJ, Yoo BC, Kragler F (2001) RNA as a long-distance information macromolecule in plants. Nat Rev Mol Cell Biol **2:** 849–857

Mathews DH, Sabina J, Zuker M, Turner DH (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol **288:** 911–940

Matsuda Y, Liang G, Zhu Y, Ma F, Nelson RS, Ding B (2002) The *Commelina yellow mottle virus* promoter drives companion cells-specific gene expression in multiple organs of transgenic tobacco. Protoplasma (in press)

Oparka KJ, Santa Cruz S (2000) The great escape: phloem transport and unloading of macromolecules. Annu Rev Plant Physiol Plant Mol Biol **51:** 323–347

Oparka KJ, Turgeon R (1999) Sieve elements and companion cells-traffic control centers of the phloem. Plant Cell **11:** 739–750

Oparka KJ, Roberts AG, Boevink P, Santa Cruz S, Roberts I, Pradel KS, Imlau A, Kotlizky G, Sauer N, Epel B (1999) Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. Cell **97:** 743–754

Owens RA, Blackburn M, Ding B (2001) Possible involvement of phloem protein 2 in long distance viroid movement. Mol Plant-Microbe Interact **14:** 905–909

Owens RA, Hammond RW, Gardner RC, Kiefer MC, Thompson SM, Cress DE (1986) Site-specific mutagenesis of potato spindle tuber viroid cDNA. Plant Mol Biol **6:** 179–192

Palauqui JC, Elmayan T, Pollien JM, Vaucheret H (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J **16:** 4738–4745

Palukaitis P (1987) Potato spindle tuber viroid: investigation of the long-distance, intra-plant transport route. Virology **158:** 239–241

Restrepo MA, Freed DD, Carrington JC (1990) Nuclear transport of plant potyviral proteins. Plant Cell **2:** 987–988

Riesner D, Gross HJ (1985) Viroids. Annu Rev Biochem **54:** 531–564

Roberts AG, Santa Cruz S, Roberts IM, Prior DAM, Turgeon R, Oparka KJ (1997) Phloem unloading in sink leaves of *Nicotiana benthamiana*: comparison of a fluorescent solute with a fluorescent virus. Plant Cell **9:** 1381–1396

Ruiz-Medrano R, Xoconostle-Cázares B, Lucas WJ (1999) Phloem long-distance transport of CmNACP mRNA: implications for supracellular regulation in plants. Development **126:** 4405–4419

Schnölzer M, Haas B, Ramm K, Hofmann H, Sänger HL (1985) Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). EMBO J **4:** 2181–2190

Szewczak AA, Moore PB (1995) The sarcin/ricin loop, a modular RNA. J Mol Biol **247:** 81–98

Ueki S, Citovsky V (2001) RNA commutes to work: regulation of plant gene expression by systemically transported RNA molecules. Bioessays **23:** 1087–1090

Voinnet O, Baulcombe DC (1997) Systemic signalling in gene silencing. Nature **389:** 553

Voinnet O, Vain P, Angell S, Baulcombe DC (1998) Systemic spread of sequence-specific transgene RNA degradation in plants is inhibited by localized introduction of ectopic promoterless DNA. Cell **95:** 177–187

Wassenegger M, Heimes S, Riedel L, Sänger HL (1994) RNA-directed de novo methylation of genomic sequences in plants. Cell **76:** 567–576

Wassenegger M, Spieker RL, Thalmeir S, Gast F-U, Riedel L, Sänger HL (1996) A single nucleotide substitution converts potato spindle tuber viroid (PSTVd) from a noninfectious to an infectious RNA for *Nicotiana tabacum*. Virology **226:** 191–197

Wimberly B, Varani G, Tinoco I Jr (1993) The conformation of loop E of eukaryotic 5S ribosomal RNA. Biochemistry **32:** 1078–1087

Woo Y-M, Itaya A, Owens RA, Tang L, Hammond RW, Chou H-C, Lai MMC, Ding B (1999) Characterization of nuclear import of potato spindle tuber viroid RNA in permeabilized protoplasts. Plant J **17:** 627–635

Wu X, Weigel D, Wigge PA (2002) Signaling in plants by intercellular RNA and protein movement. Genes Dev **16:** 151–158

Zambryski P, Crawford K (2000) Plasmodesmata: gatekeepers for cell-to-cell transport of developmental signals in plants. Annu Rev Cell Dev Biol **16:** 393–421

Zhao Y, Owens R, Hammond R (2001) Use of a potato virus X vector in a whole plant assay to demonstrate nuclear targeting of potato spindle tuber viroid. J Gen Virol **82:** 1491–1497

Zhu Y, Green L, Woo Y-M, Owens RA, Ding B (2001) Cellular basis of potato spindle tuber viroid systemic movement. Virology **279:** 69–77

Zuker M, Mathews DH, Turner DH (1999) Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. *In* RNA Biochemistry and Biotechnology. NATO ASI Series, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 11–43