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Intracellular and Intercellular Transport of Viroids

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Abstract Viroids are small, noncoding and nonencapsidated RNAs that infect plants. To establish systemic infection, viroid genomes or their derivatives must interact directly with cellular factors. There is increasing evidence that subcellular localization and systemic trafficking of viroid RNAs are regulated, likely via interactions between viroid RNA *cis* elements and specific cellular proteins. Here we summarize recent progress on the characterization of viroid structures and host proteins that may play important roles in trafficking. We also discuss critical issues that need to be addressed in future investigations.

1 Introduction

Viroids are small (250–400 nt), single-stranded and circular RNAs that infect plants. Approximately 40 species of viroids are known to date and they are classified into two families: The Pospiviroidae family that replicates in the nucleus and the Avsunviroidae family that replicates in the chloroplasts (Tabler and Tsagris 2004; Góra-Sochacka 2004; Flores et al. 2000, 2005). The systemic infection process of viroids encompasses several distinguishable steps: import into the nucleus (the Pospiviroidae) and chloroplasts (the Avsunviroidae), replication within these organelles, export out of these organelles, cell-to-cell movement, entry into the vascular tissue, long distance transport within the vascular tissue, and finally exit from the vascular tissue to infect neighboring cells (Ding et al. 2005). Unlike viruses, viroid RNAs have no protein-coding capacity and are not encapsidated within protein or membrane shells. Therefore, these RNAs most likely utilize pre-existing cellular machineries for systemic trafficking to establish infection. In the simplest scenario, virioid RNA can be envisaged to have evolved distinct *cis* elements that interact with specific cellular factors to accomplish trafficking within a cell, from cell to cell, and from organ to organ to establish systemic infection.

Viroid trafficking is still a rather fresh field with many fundamental questions unanswered and new research tools to be developed. Yet the small size of the RNAs, their experimental tractability and reliance on host factors

for trafficking make viroids attractive tools for uncovering the basic plant mechanisms that regulate RNA trafficking within and between cells (Ding et al. 2005). Here we highlight recent progress on the characterization of viroid structures and host proteins that may play important roles in trafficking. We also discuss critical issues that need to be addressed in future investigations.

2
Organellar Import and Export of Viroid RNAs

Despite their pivotal importance for the initiation of viroid replication, how organellar import of viroid RNAs occurs in a cell is poorly understood. Two approaches have been developed to investigate nuclear import of Potato spindle tuber viroid (PSTVd). In a cellular approach, Woo et al. (1999) characterized nuclear import of fluorescently labeled PSTVd in vitro transcripts in permeabilized tobacco BY2 protoplasts. They showed that import of these transcripts could be inhibited specifically in the presence of nonlabeled transcripts of PSTVd (Woo et al. 1999). In a molecular approach, Zhao et al. (2001) showed that PSTVd could act in *cis* to direct nuclear import of a fusion RNA in Nicotiana benthamiana leaves. All of these results imply the existence of a *cis* element in PSTVd that serves as a nuclear import signal. This signal, as well as the cellular factors that recognize this signal, remain unknown. Nuclear export of any viroid RNAs has not been explored.

Mechanisms for the chloroplastic import or export of viroids in the Avsunviroidae are not understood. It may be speculated that these viroids utilize a pre-existing system for entering and exiting the chloroplasts. Elucidating the underlying mechanisms may have broad ramifications in the basic biology of chloroplasts and in chloroplast–cytoplasm communications.

3
Strand Polarities of Viroid RNAs Control Subcellular Localization

PSTVd replicates via an asymmetric rolling circle in which RNAs of the (+)- and (–)-strand polarities are produced (Branch and Robertson 1984; Fig. 1). Based on fluorescence in situ hybridization studies, Qi and Ding (2003) showed that the (–)-strand PSTVd RNAs were localized in the nucleoplasm whereas the (+)-strand PSTVd RNAs were present in the nucleolus as well as the nucleoplasm. These results suggest that the (+)-PSTVd RNAs synthesized in the nucleoplasm are selectively transported into the nucleolus. It is likely that these RNAs contain a *cis* element, which remains to be identified, that directs their nucleolar import. The nucleoplasmic localization of the (–)-strand PSTVd RNAs may be attributed to the lack of a nucleolar

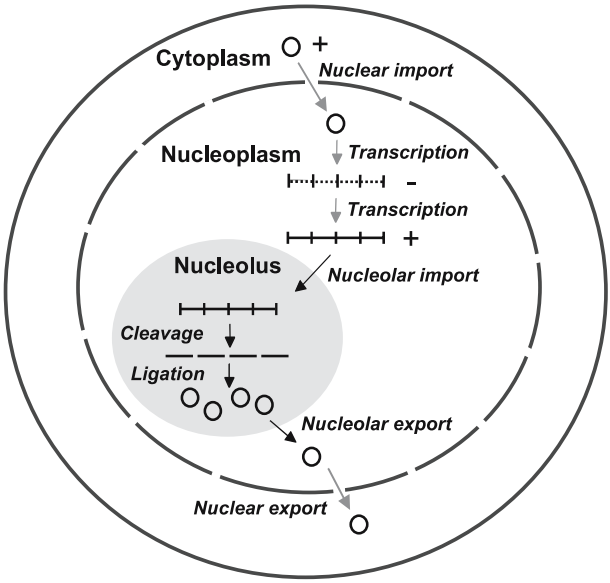

Fig. 1 Model for the replication of PSTVd. The incoming (+)-circular genomic RNA is imported from the cytoplasm into the nucleus, where it is transcribed into concatemeric linear (–)-strand RNAs, which then serve as the replication intermediate to synthesize concatemeric, linear (+)-strand RNAs. These (+)-strand RNAs are subsequently imported into the nucleolus for cleavage and ligation to form the circular molecules. Some circular molecules are exported out of the nucleolus and further out of the nucleus to enter the cytoplasm. Some of these molecules traffic into neighboring cells (not shown) to embark on systemic infection. (Modified from Qi and Ding 2003)

import signal, presence of a nucleoplasmic localization signal, or both. Elucidating the viroid RNA *cis* elements as well as the cellular factors that are responsible for the distinct subnuclear localization patterns will be essential to understand the infection of viroids in the Pospiviroidae family. It will also generate fundamental knowledge about subnuclear trafficking/localization of cellular RNAs.

4 Polarized Subnucleolar Localization of PSTVd RNAs

Detailed examination of PSTVd localization within the nucleolus in comparison with that of some small nucleolar RNAs (snoRNAs) revealed striking patterns of polarized localization (Qi and Ding 2003). Previous studies showed that U3 snoRNA was localized evenly in subnucleolar compartments (Beven et al. 1996). Strikingly, in over 10% of the infected cells, the (+)-PSTVd RNA and U3 were localized in separate domains within the nucleolus. In

each case when the (+)-PSTVd was localized in one part of the nucleolus, the U3 snoRNA was localized in the remaining part. Thus, the (+)-PSTVd RNA may compete with U3 snoRNA for certain nucleolar factors, with the consequence that the U3 is displaced from a part of its normal nucleolar domain. Another small nucleolar RNA, U14, also exhibited similar redistribution within the nucleolus in the presence of (+)-PSTVd in some cells. The significance of this polarized localization of PSTVd RNAs in relation to snoRNAs remains to be understood.

5
Viroid RNA *Cis* Elements Mediate Cell-to-Cell Trafficking

When fluorescently labeled PSTVd RNA transcripts were injected into mesophyll cells, they moved rapidly into neighboring cells. In contrast, the transcripts injected into symplasmically isolated guard cells were retained in such cells. These findings provided strong evidence that PSTVd traffics through plasmodesmata (Ding et al. 1997). Cell-to-cell trafficking of PSTVd RNA between mesophyll cells does not appear to occur by diffusion, but is directed by *cis* elements residing in the RNA. The evidence came from the observation that PSTVd can mediate trafficking of a heterologous fusion RNA (Ding et al. 1997).

Recent studies provided genetic evidence for the existence of *cis* elements in regulating unidirectional trafficking of PSTVd between specific cell types. Two PSTVd strains, $PSTVd^{NT}$ and $PSTVd^{NB}$, differ by five nucleotides. Using in situ hybridization, Qi et al. (2004) showed that in young systemically infected tobacco leaves, $PSTVd^{NB}$ was detected in all types of cells. In contrast, $PSTVd^{NT}$ is found only in the phloem and bundle sheath cells. This difference in the distribution patterns is attributed to the inability of $PSTVd^{NT}$ to traffic out of the bundle sheath cells. Interestingly, when a leaf matures to certain stage, $PSTVd^{NT}$ can move into mesophyll cells (Qi et al. 2004). Mutational studies showed that four of the five $PSTVd^{NB}$-specific nucleotides are all required and sufficient to potentiate trafficking of $PSTVd^{NB}$ from bundle sheath into the mesophyll. Because $PSTVd^{NT}$ introduced into epidermal cells by rub-inoculation or biolistic bombardment could establish systemic infection, the four $PSTVd^{NB}$-specific nucleotides are not required for trafficking from epidermal cells into the phloem for systemic spread. Thus, trafficking between the bundle sheath and mesophyll in opposite directions is differently regulated, likely involving different RNA motifs (Qi et al. 2004; Fig. 2). *Cis* elements that remain to be identified and plant development also appear to regulate trafficking of cellular RNAs (Haywood et al. 2005).

Altogether, these studies lead to a model in which cell-to-cell trafficking of an RNA is mediated by specific *cis* elements. Furthermore, results from PSTVd analyses indicate that trafficking of an RNA across different cellu-

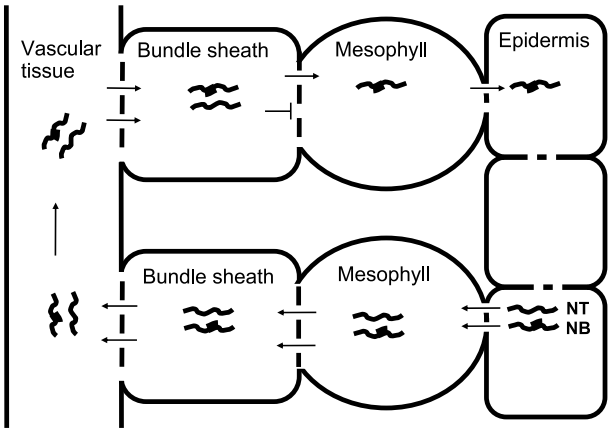

Fig. 2 *Cis* element-mediated unidirectional trafficking of PSTVd across bundle sheath–mesophyll boundary. When inoculated into an epidermal cell in a young tobacco leaf, both $PSTVd^{NT}$ and $PSTVd^{NB}$ replicate and traffic through mesophyll, bundle sheath, and finally into the vascular tissue for long distance transport. In a systemically infected young leaf, both PSTVd stains exit the vascular tissue to enter bundle sheath. While $PSTVd^{NB}$ traffics further into all cell types to establish infection, $PSTVd^{NT}$ remains in the bundle sheath due to inhibited trafficking into the mesophyll. The *rectangle mark on the wavy bar* indicative of $PSTVd^{NB}$ represents the *cis* element that mediates trafficking from the bundle sheath to the mesophyll. *Arrows* show the directions of trafficking. (Based on Qi et al. 2004)

lar boundaries and in opposite directions are uniquely regulated (Ding et al. 2005).

6 Long-Distance Trafficking of Viroid RNAs

Palukaitis (1987) reported the first study on the long distance trafficking of viroid RNAs. His analysis of the systemic infection patterns of PSTVd in tomato in comparison with the known source-to-sink transport patterns of photoassimilates indicated that PSTVd is transported within the phloem. Zhu et al. (2001) used in situ hybridization to localize PSTVd to the phloem, providing cellular evidence for the phloem pathway for long distance transport. In the shoot apices, PSTVd is selectively transported into sepals but not into the other floral organs (Zhu et al. 2001, 2002). Because all floral organs are strong sinks for photoassimilates, these observations imply that a phloem-based mechanism recognizes PSTVd and transports it into the sepals. It is also possible that another phloem-based mechanism actively prevents PSTVd from entering such floral organs as petals, stamen, and ovary. Importantly, phloem-based mechanisms also appear to transport cellular RNAs (Haywood

et al. 2005) and proteins (Aoki et al. 2005) to selective sink organs. Thus, further studies on the long distance trafficking of viroid RNAs should provide useful insights into the basic mechanisms of phloem-mediated transport of a wide range of macromolecules.

7
Cellular Factors that May Facilitate Viroid RNA Trafficking

Because *cis* elements apparently play a critical role in mediating trafficking of viroid RNAs, such *cis* elements may well interact with cellular factors. Such factors remain unknown. However, recent work has revealed a number of cellular proteins as promising candidate factors for viroid trafficking. The first is the phloem lectin PP2 from cucumber (CsPP2), which binds Hop stunt viroid (HSVd) in vitro (Gómez and Pallás 2001; Owens et al. 2001). However, the binding is not specific to HSVd, because the protein also binds a viral RNA (Owens et al. 2001). Recent co-immunoprecipitation experiments demonstrated in vivo interactions between CsPP2 and HSVd in infected cucumber plants (Gómez and Pallás 2004), providing evidence for the functional significance of the interactions. In support of this notion, CsPP2 (Golecki et al. 1999; Gómez and Pallás 2004) and HSVd (Gómez and Pallás 2004) can both traffic from rootstocks into scions in heterografts and the CsPP2 has an RNA-binding motif (Gómez and Pallás 2004).

The tomato protein VIRP1 (Martinez de Alba et al. 2000) interacts with the right-terminal regions of PSTVd and HSVd (Maniataki et al. 2003; Gozmanova et al. 2003). Specifically, the interaction involves two asymmetric internal loop motifs of PSTVd (Gozmanova et al. 2003). This interaction may be important for infection because mutations in these motifs that disrupt VIRP1 binding inhibit infection (Gozmanova et al. 2003). Other mutations in the right-terminal domain of PSTVd that abolish infection of tomato by mechanical inoculation but not by agroinoculation (Hammond 1994) also compromise interactions with VIRP1, suggesting that the interaction between VIRP1 and the right-terminal domain of PSTVd is likely important for trafficking (Maniataki et al. 2003).

It is also of great interest that candidate proteins that may play a role in the trafficking of viroids of the Avsunviroidae family have emerged. Gómez et al. (2005) recently found two proteins that bind Avocado sunblotch viroid (ASBVd). One is the previously characterized CmmLec17 (Dinant et al. 2003) and the other a 14 kDa protein. The CmmLec17 moves long distances from rootstocks to scions in heterografts, raising the possibility that this protein may be involved in ASBVd trafficking.

In summary, some strong candidate cellular proteins for viroid trafficking have been uncovered. Conclusive evidence for their role in viroid trafficking will await further genetic and molecular studies.

8
Future Prospects

Recent development in methodology has allowed rapid progress in the characterization of viroid RNA *cis* elements and cellular proteins that may play crucial roles in the subcellular and systemic trafficking of viroids from both the Pospiviroidae and Avsunviroidae families. A clear future goal in this area is to further determine the nature of these *cis* elements and the cellular proteins that interact with these elements. Importantly, because distinct *cis* elements might be involved in trafficking between specific cells and in a particular direction, it follows that cell-specific factors may be involved. Elucidating these *cis* elements and the cognate cellular factors should help establish a solid conceptual framework for investigating the regulated trafficking of viroid, viral and cellular RNAs.

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