Running title

Intracellular traffic of a non coding-pathogenic RNA

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Title:

Studies on subcellular compartmentalization of plant pathogenic non coding RNAs give new insights into the intracellular RNA-traffic mechanisms

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The subcellular localization and trafficking of cellular RNAs are essential processes governing plant growth and development. RNAs are targeted to specific regions/organelles mainly by active transport that requires RNA zip codes that are recognized by specific RNA binding activities, accessory proteins such as adaptors for cytoskeletal movement and, in some instances, components for translation (Okita and Choi, 2002; Michaud et al., 2010). Unlike the progress made in studies on cell-to-cell and long-distance RNA trafficking (Fernandez-Calvino et al., 2011; Pallas et al., 2011; Gomez and Pallas, 2004; Lucas et al., 2001), our understanding of intracellular RNA trafficking in plants remains limited.

Viroids, the smallest non-coding RNAs able to subvert plant cell metabolism to induce pathogenesis in their hosts, have recently emerged as ideal probes for exploring intracellular RNA trafficking (Wang and Ding, 2010). Viroids are single-stranded circular RNAs ranging in size from 250 to 400 nt with a high degree of self-complementarity that promotes compact folding (Ding, 2009; Flores et al., 2005; Tabler and Tsagris, 2004). Lacking mRNA activity, these pathogenic RNAs are completely dependent on functional genomic domains that interact with host factors to complete their life cycle in the infected cell (Diener, 1987). Viroids belong to one of two families: *Pospiviroidae*, the replication of which takes place in the nucleus, and *Avsunviroidae*, whose members possess ribozyme activity and replicate in the chloroplasts of infected cells (Ding, 2009; Flores et al., 2005; Tabler and Tsagris, 2004).

Upon mechanical introduction into a susceptible cell, viroid infection comprises a series of sequential events: a) subcellular compartmentalization for replication, b) export to neighboring cells and, c) entry to vascular tissue for long-distance trafficking and to establish systemic infection ((Ding, 2009; Flores et al., 2005). In these circumstances, trafficking into cellular organelles (chloroplasts for *Avsunviroidae* and nucleus for *Pospiviroidae*) is the first hurdle to be overcome by the viroids after infecting the host. Consequently, elucidating how these non-coding RNAs are trafficked to the nucleus and chloroplasts to initiate their replication is fundamental to fully understanding the biology of these RNAs. Furthermore, such knowledge would yield insights into the poorly understood mechanisms of intracellular localization of RNAs in plant cells (Ding and Itaya, 2007).

Initial evidence suggested that the subcellular localization of viroids in the *Pospiviroidae* family is mediated in *cis* by RNA sequences or structural motifs, which are required for nuclear import by a specific receptor via a cytoskeleton-independent route (Abraitiene et al., 2008; Zhao et al., 2001; Woo et al., 1999). However, how members of the *Avsunviroidae* are translocated into the chloroplast is an intriguing and challenging question because they are the only known pathogenic-RNAs able to traffic into this organelle (Wang and Ding, 2010; Daròs et al., 2006). Although the mechanism of import of RNAs into chloroplasts has previously been suggested for both nuclear encoded tRNAs (Bungard, 2004) and the mRNA coding for the translation factor eIF4E (Nicolai et al., 2007), the plant-cell endogenous mechanisms for RNA import that are exploited by viroids when entering chloroplasts remains unknown.

In recent work we demonstrated that a chimeric (containing partial length *plus* and *minus* RNA) sequence derived from the ELVd *(Eggplant latent viroid* – a member of the *Avsunviroidae* family) acting as the 5′UTR-end was able to mediate the specific trafficking and accumulation of functional GFP mRNA from the nucleus into chloroplasts (Gomez and Pallas, 2010a; 2010b). This formed the experimental basis for proposing the existence of a novel plant signaling mechanism (mediated by non-coding RNAs) able to regulate the selective import of nuclear transcripts into chloroplasts. Furthermore, we speculated that *Avsunviroidae* members may subvert this signaling mechanism to mediate their specific trafficking to this organelle. However, this pathway requires the existence of a previous step involving the transport of the viroid from the cytoplasm to the nucleus, such that during the initial phase of their life cycle the *Avsunviroidae* would be first transported from the cytoplasm into the nucleus and then specifically delivered to the chloroplasts, where they replicate.

To determine whether this RNA import pathway is used by members of the *Avsunviroidae* family for transit to the chloroplasts, we analyzed the main stages in this route. First, we developed a *Potato virus X* (PVX)-based *in vivo* assay that used a [Green Fluorescent protein (GFP)-intron-ELVd] construct as a reporter to determine whether this RNA is able to traffic from the cytoplasm to the nucleus. Next, we used an engineered reporter containing the same full-length ELVd sequence fused as the 5′UTR-end to the GFP cDNA. This construct was used in transient expression assays to confirm the selective trafficking of canonical ELVd monomeric sequence from the nucleus into the chloroplast. The results shown here provide evidence that in members of the family *Avsunviroidae*, selective intracellular trafficking is a complex process comprising a first nuclear step prior to their delivery to the chloroplast of the infected cell.

ELVd-RNA moves from cytoplasm to nucleus

To determine whether ELVd is able to traffic into the nucleus of the host cell, we used an experimental approach similar to that previously employed to demonstrate the selective import of PSTVd into the nucleus of *N. benthamiana* cells (Abraitiene et al., 2008; Zhao et al., 2001). First, the coding region of the GFP was interrupted by the insertion of an intron (IV2) derived from the potato (*S. tuberosum*) ST-LS1 gene (Eckes et al., 1986). The monomeric plus-strand of the full-length ELVd sequence (Fadda et al., 2003) was subsequently inserted into the intron to generate an IV2/ELVd-bearing GFP construct (Supplemental file 1). This reporter was cloned in a PVX-based vector (pP2C2S) (Chapman et al., 1992) (Figure 1A). These constructs were transcribed *in vitro* and used to inoculate *N. benthamiana* leaves. During PVX replication the different reporters used in this assay were expressed in the cytoplasm of the infected cells.

When leaves infected with the PVX carrying the IV2/ELVd(*plus*)-bearing GFP construct were analyzed at 7 and 10 days post-inoculation (Figure 1B), we observed fluorescence resembling that observed in the *N. benthamiana* leaves inoculated with the PVX-GFP control. As shown in Figure 1B, although GFP was readily visible throughout the leaves, the fluorescence was more intense in vascular tissues. No GFP expression was

observed in the *N. benthamiana* leaves inoculated with the PVX carrying the IV2-bearing GFP construct. GFP expression in the infected leaves was corroborated by Western blot assays. As PVX replication occurs in the cytoplasm of the infected cell, the sub-genomic transcripts containing the intron are not processed and translated to produce functional GFP (as observed in the control PVX/IV2-bearing GFP). Consequently the fluorescence observed in the leaves infected with the PVX carrying the IV2/ELVd(*plus*)-bearing GFP construct could only be explained by assuming that this cytoplasmatic transcript is directed by the ELVd-RNA to the nucleus, where the intron can be efficiently removed to generate a functional GFP mRNA that is later translated.

In order to confirm the correct processing and stability of the constructs in the PVX-infected plants we analyzed the sub-genomic transcripts found in systemically infected *N. benthamiana* leaves by RT-PCR and sequencing. On amplification of the transcripts with GFP-specific primers, we observed a product of 724 bp corresponding to full GFP cDNA in the leaves inoculated with the PVX/GFP and PVX-GFP/IV2/ELVd(+)/constructs, providing evidence that the intron was correctly removed in the ELVd(+)-containing construct. An upper band (~1250 bp) corresponding to residual non-spliced mRNA was observed in the leaves inoculated with the PVX-GFP/IV2/ELVd(+) transcripts, suggesting that the import of mRNA into the nucleus was not fully efficient. As expected, the amplification product corresponding to non-spliced mRNA was the only band observed in the leaves inoculated with PVX-GFP/IV2 transcripts (Figure 1D).

To rule out the possibility that the insertion IV2/ELVd(+) was eliminated from the chimeric PVX during long-distance movement, thereby yielding functional GFP, we assessed the presence of ELVd and IV2 RNA in these leaves at 25 days post-inoculation. As shown in Figure 2, both insertions were detected in leaves systemically infected with the constructs PVX-GFP/IV2 and PVX-GFP/IV2/ELVd(+), indicating that the chimeric PVX maintained its stability during their translocation. Sequencing of the amplified cDNAs corroborated the correct processing and stability of the analyzed transcripts (Supplemental Figure 1).

To obtain a more detailed picture, we dissected the ELVd-RNA into three arbitrary regions identified as **L** (Left), **R** (Right) and **Up** (Upper) (Supplemental Figure 2). Subsequently, these partial-length sequences were inserted into the PVX carrying the IV2/bearing GFP vector to generate the PVX-GFP/IV2/L-ELVd, PVX-GFP/IV2/R-ELVd and PVX-GFP/IV2/Up-ELVd constructs. The *N. benthamiana* plants inoculated with the transcripts derived from these infectious clones were analyzed at 6, 9 and 11 dpi. We observed that the leaves infected with the PVX-GFP/IV2/L-ELVd transcripts exhibit fluorescence resembling that observed in the plants inoculated with the PVX-GFP control (Figure 3A). However, no GFP expression was observed in the *N. benthamiana* leaves infected with the PVX-GFP/IV2/R-ELVd and PVX-GFP/IV2/Up-ELVd constructs. This finding reveals that the **L** region of the ELVd is sufficient to mediate the traffic of GFP-mRNA into the nucleus, indicating that the nuclear specific signal is localized in this 168 nt-length ELVd-RNA sequence. The correct processing of the chimeric constructs in the infected plants was analyzed by RT-PCR (Figure 3C).

Monomeric ELVd RNA is specifically delivered from the nucleus to chloroplasts

Having established that the monomeric linear form (+) of the ELVd RNA expressed in the cytoplasm is efficiently imported into the nucleus, we investigated whether this biological form of the ELVd can be delivered from the nucleus to its specific replication site in the chloroplast. To address this issue we constructed (Supplemental File 1) a reporter containing a monomeric cDNA derived from the genomic (+)ELVd RNA sequence fused as an untranslated region (UTR) to the 5ꞌ-end of the cDNA of GFP (Figure 4A and Supplemental Figure 3). The fused ELVd-GFP cDNA (ELVd-5ꞌUTR-GFP) was cloned in a binary vector to be transfected into *Agrobacterium*. This construct was analyzed by transient expression in *N. benthamiana* plants by means of agro-infiltration assays. First, we evaluated GFP expression in agro-infiltrated leaves by Western blot. As shown in Figure 4B the ELVd-5ꞌUTR-GFP exhibited a relative electrophoretic mobility identical to the unmodified GFP used as the control, indicating that the ELVd RNA acts as a true 5ꞌ-UTR in this GFP mRNA. Analysis of the infiltrated plants by confocal microscopy revealed that the GFP arising from the transcripts ELVd-5´UTR-GFP was generally concentrated in the chloroplasts of the *N. benthamiana* cells, resembling the localization of the GFP fused to a chloroplast-specific transit peptide OE23 (Roffey and Theg, 1996) used as a control (Figure 4C). These results provide direct evidence that the ELVd-5ꞌUTR-GFP mRNA transcribed in the nucleus is specifically targeted to chloroplasts where it is efficiently retained and translated into functional GFP. This finding confirms that, in a similar way to that observed for a chimeric (*plus* and *minus*) ELVd-RNA (Gomez and Pallas, 2010a), the biological monomeric form of this *Avsunviroidae* contains sufficient information in its genomic RNA to regulate its specific translocation from the nucleus to the chloroplasts of the infected cell.

Discussion

Recent evidence indicates that it is not only proteins that possess internal localization signals for their targeting to specific destinations, but that RNAs may also harbor appropriate motifs to ensure intracellular trafficking to the appropriate destination (Okita and Choi, 2002; Nevo-Dinur et al., 2011; Rymarquis et al., 2008). Because viroids lack protein coding capacity they provide an ideal model system for studying intracellular RNA transport in plants (Wang and Ding, 2010; DiSerio and Flores, 2008). How the members of the *Avsunviroidae* family traffic into chloroplasts of infected cells to replicate remains a fascinating question that, once elucidated, will advance our understanding of viroid biology and intracellular RNA trafficking in general (Ding, 2009). According to these premises, and employing as a starting point the observation that a chimeric RNA containing partial sequences derived from both (*plus* and *minus*) ELVd forms regulates the selective trafficking of a functional mRNA from the nucleus into chloroplasts (Gomez and Pallas, 2010a; 2010b), we used a combined approach involving the cytoplasmic and nuclear expression of the biological monomeric form

(*plus* strand) of the ELVd RNA to study relevant aspects of their intracellular movement *in vivo*.

In eukaryotic organisms intron splicing is a nucleus-specific process catalyzed by the spliceosome, a large ribonucleoprotein complex comprising several small nuclear RNAs (snRNAs), small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP protein splicing factors (Sperling et al., 2008). Consequently, the observation that the GFP mRNA arising from the PVX-GFP/IV2/ELVd(+) construct is translated into functional fluorescent protein provides strong evidence supporting the nuclear import of the viroid. Since PVX replicates exclusively in the cytoplasm, the mRNA carrying the intron IV2 will not produce a functional GFP unless this RNA can be targeted by the ELVd-RNA to the nucleus, where the intron can be efficiently removed. This notion is consistent with previous reports showing that minor fractions of the ASBVd *(Avocado sunblotch viroid)* (Mohamed and Thomas, 1980; Marcos and Flores, 1990) and PLMVd *(Peach latent viroid)* (Bussiere et al., 1999) (also members of *Avsunviroidae* family) could be detected in the nuclei of the infected cells. Furthermore, the recent observation that the ASBVd could be maintained in the nucleus of the yeast *S. cerevisiae* for 25 generations (Delan-Fiorino et al., 2011) provides additional evidence supporting the potential robustness of chloroplastic viroids to survive in a nuclear environment.

The findings shown suggest that the capacity of a linear monomeric sequence to regulate its nuclear targeting mimics an intrinsic characteristic of the circular mature form of the ELVd and that this nuclear stage could occur in the infected cell. It is worth noting that cell-to-cell movement (Ding et al., 1997) and nuclear-specific compartmentalization (Zhao et al., 2001), both natural abilities of the circular form, were retained by linearized PSTVd-RNA fused to reporter sequences. Furthermore, our results indicate that the structural and/or sequence domain involved in this signaling mechanism is localized in a specific region comprised between the positions 16 and 182 of the monomeric ELVd-cDNA.

Having demonstrated the potential nuclear phase of the viroid RNA, we developed a differential construct to determine whether the monomeric ELVd (plus strand) was able to traffic from the nucleus to the chloroplast. The observation that the GFP arising from the transcript (5′UTR-ELVd-GFP) accumulated specifically in the chloroplasts of the agro-infiltrated plant cells indicates that the ELVd genomic RNA (acting as untranslated sequence in this transcript) is selectively delivered from the nucleus into chloroplasts, providing biological evidence that, once localized in the nucleus, the monomeric form of the ELVd is able to hijack endogenous routes for the export of nuclear RNAs to the chloroplast. This novel and uncharacterized (nucleus–chloroplast) RNA signaling pathway, which was recently proposed as an alternative mechanism to regulate the accumulation of nucleus-encoded proteins lacking canonical transit peptides in chloroplasts (Gomez and Pallas, 2010a), is consistent with previous reports showing that other endogenous RNAs are also targeted to this organelle in Chlamydomonas (Uniacke and Zerges, 2009) and plant cells (Bungard, 2004; Nicolai et al., 2007). Furthermore, the recent observation that the *Alternanthera mosaic virus* RNA localizes around chloroplast membranes in infected cells (Lim et al., 2010),

suggest that the association between viral RNA and chloroplasts could have functional implication in the infectious process.

Recent results have shown that the specific trafficking of partial-length ELVd-transcripts fused to GFP mRNA viroid from the nucleus to the chloroplast is mediated by a potential structural domain contained in a short fragment localized between position 52 and 150 of the ELVd cDNA (Gomez and Pallas, 2010b). We note that this fragment involved in the chloroplastic compartmentalization of the viroid overlaps with the region described here as responsible for the nuclear localization of the ELVd (between nucleotides 15 and 181), suggesting that a RNA sequence and/or structure, restricted to the left terminal region of the ELVd, can mediate both subsequent nuclear and chloroplastic localization. Interestingly, it has previously been shown that an ELVd mutant carrying a deletion in this region (identified as ELVd-D7) failed to infect the natural host eggplant even though it is processed (cleavage and ligation) *in vivo* in a chloroplastic context using the *C. reinhardtii*-based experimental system (Martinez et al., 2009). This observation suggests that the deleted region is involved in other essential functions in the ELVd life-cycle, for example chloroplastic localization. Interestingly, the possibility that members of the *Avsunviroidae* family have evolved a specific motif that is recognized by the cell machinery for an as yet undiscovered RNA import mechanism into the chloroplast was previously suggested as a potential way to explain their selective intracellular localization (Ding and Itaya, 2007).

In summary, the findings shown here provide insights into aspects of the intracellular movement of viroids belonging to the *Avsunviroidae* family. The observation that the monomeric linear form of the ELVd possesses the potential to selectively traffic from the cytoplasm into the nucleus and subsequently from this organelle into chloroplasts allows us to envision a novel route that could explain their selective compartmentalization in the chloroplast. In this hypothetical pathway (likely mediated by a RNA domain localized in the left terminal region), after the viroid (mainly the plus-strand circular form) is mechanically introduced into the cell cytoplasm, it is imported into the nucleus, by means of an unknown host-dependent mechanism. The viroid then uses this organelle as a sub-cellular shuttle for delivery into the chloroplast, where replication takes place. Our results reveal that the different steps of this pathway take place in the plant, and highlight the fact that viroids can be used to help unravel the trafficking of RNAs to specific destinations in the cell.

References

Abraitiene A, Zhao Y, Hammond R (2008) Nuclear targeting by fragmentation of the potato spindle tuber viroid genome. *Biochem Biophys Res Commun* 368: 470-475.

Bungard R-A (2004) Photosynthetic evolution in parasitic plants: insight from the chloroplast genome. *Bioessays* 26: 235-247.

Bussière F, Lehoux J, Thompson D-A, Skrzeczkowski L-J, Perreault J (1999) Sub-cellular localization and rolling circle replication of peach latent mosaic viroid: hallmarks of group A viroids. *J Virol* 73: 6353-6360.

Chapman S, Kavanagh T, Baulcombe D (1992) Potato virus X as a vector for gene expression in plants. *Plant J* 2: 549-557

Daròs J-A, Elena S-F, Flores R (2006) Viroids: an Ariadne's thread into the RNA labyrinth. *EMBO Rep* 7: 593-598.

Delan-Forino C, Maurel M-C, Torchet C (2011) Replication of *Avocado sunblotch viroid* in the yeast *Saccharomyces cerevisiae*. *J Virol* 85: 3229-3238

Di Serio F, Flores R (2008) Viroids: molecular implements for dissecting RNA trafficking in plants. *RNA Biol* 5: 128-131.

Diener T-O (1987) The Viroids. New York, Plenum Press.

Ding B (2009) The biology of viroid-host interactions. *Annu Rev Phytopathol* 47: 105-131.

Ding B, Itaya A (2007) Viroid: a useful model for studying the basic principles of infection and RNA biology. *Mol Plant Microbe Interact* 20: 7-20.

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

Eckes P, Rosahl S, Schell J, Willmitzer L (1986) Isolation and characterization of a light-inducible, organ-specific gene from potato and analysis of its expression after tagging and transfer into tobacco and potato shoots. *Mol Gen Genetics* 205: 14-22.

Fadda Z, Daròs J-A, Fagoaga C, Flores R, Duran-Vila N (2003) Eggplant latent viroid, the candidate type species for a new genus within the family Avsunviroidae. *J Virol* 77: 6528-6532.

Fernandez Calvino L, Faulkner C, Maule A (2011) Plasmodesmata as active conduits for virus cell-to-cell movement. In *Recent Advances in Plant Virology*, eds Caranta C, Aranda M-A, Tepfer M, Lopez Moya J-J (Caister Academic Press, Norkfolk), pp 47-74.

Flores R, Hernández C, Martínez de Alba A-E, Daròs J-A, Di Serio F (2005) Viroids and viroid-host interactions. *Annu Rev Phytopathol* 43: 117-139.

Gómez G, Pallás V (2004) A long distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex in vivo with Hop stunt viroid RNA. *J Virol* 78: 10104-10110.

Gómez G, Pallás V (2007) Mature monomeric forms of Hop stunt viroid resist RNA silencing in transgenic plants. *Plant J* 51: 1041-1049.

Gómez G, Pallás V (2010a) Noncoding RNA mediated traffic of foreign mRNA into chloroplasts reveals a novel signaling mechanism in plants. *PLoS One* 5(8):e12269.

Gómez G, Pallás, V (2010b) Can the import of mRNA into chloroplasts be mediated by a secondary structure of a small non-coding RNA? *Plant Signal Behav* 5: 1517-1519.

Lim H-S, Vaira A-M, Bae H, Brag J-N, Ruzin S-E, et al (2010) Mutation of a chloroplast-targeting signal in *Alternanthera mosaic virus* TGB3 impairs cell-to-cell movement and eliminates long distance virus movement. *J Gen Virol* 91: 2012-2115.

Lucas W-J, Yoo B-C, Kragler F (2001) RNA as a long-distance information macromolecule in plants. *Nature Rev Mol Cell Bio* 2: 849-857.

Marcos J, Flores R (1990) Subcellular localization of *Avocado sunblotch viroid* in avocado leaves. *Plant Sci* 67: 237-244

Martínez F, Marqués J, Salvador M-L, Daròs J-A (2009) Mutational analysis of eggplant latent viroid RNA processing in Chlamydomonas reinhardtii chloroplast. *J Gen Virol* 90: 3057-3065.

Michaud M, Marechal-Drouard L, Duchene A-M (2010) RNA trafficking in plant cells: targeting of cytosolic mRNAs to the mitochondrial surface. *Plant Mol Biol* 73: 697-704.

Mohamed N-A, Thomas W (1980) Viroid-like properties of an RNA species associated with the sunblotch disease of avocado. *J Gen Virol* 46: 157-167.

Nevo-Dinur K, Nussbaum-Schochat A, Ben-Yeuda S, Amster-Choder O (2011) Translation-independent localization of mRNA in *E. coli*. *Science* 331: 1081-1084.

Nicolai M, Duprat A, Sormani R, Rodriguez C, Roncato M-A, et al (2007) Higher plant chloroplasts import the mRNA coding for the eukaryotic translation initiation factor 4E. *FEBS Lett* 581: 3921-3926.

Okita T-W, Choi S-B (2002) mRNA localization in plants: targeting to the cell's cortical region and beyond. *Curr Op Plant Biol* 5: 553-559.

Pallás V, Genoves A, Sanchez-Pina M-A, Navarro J-A (2011) Systemic movement of viruses via the plant phloem. In *Recent Advances in Plant Virology*, eds Caranta C, Aranda M-A, Tepfer M, Lopez Moya J-J (Caister Academic Press, Norkfolk), pp 75-102.

Roffey R-A, Theg S-M (1996) Analysis of the import of carboxyl-terminal truncations of the 23-kilodalton subunit of the Oxygen-Evolving Complex suggests that its structure is an important determinant for thylakoid transport. *Plant Physiol* 111: 1329-1338.

Rymarquis L-A, Kastenmayer J-P, Hüttenhofer A-G, Green P-J (2008) Diamonds in the rough: mRNA-like non-coding RNAs. *Trends Plant Sci* 13:329-334.

Sperling J, Azubel M, Sperling R (2008) Structure and function of the Pre-mRNA splicing machine. *Structure* 16: 1605-1615.

Tabler M, Tsagris M (2004). Viroids: petite RNA pathogens with distinguished talents. *Trends Plant Sci* 9: 339 -348.

Uniacke J, Zerges W (2009) Chloroplast protein targeting involves localized translation in Chlamydomonas. *Proc Natl Acad Sci* USA 106: 1439-1444.

Wang Y, Ding B (2010) Viroids: small probes for exploring the vast universe of RNA trafficking in plants. *J Integr Plant Biol* 52: 28-39.

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

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

Figure Legends

Figure 1: Analysis of GFP expression in plants infected with PVX-derived transcripts. A) Physical map of the PVX-derived construct used in this work. PVX-based vector P2C2S and cDNAs (GFP, GFP/IV2 and GFP/IV2/ELVd) cloned under the control of the duplicated PVX-CP promoter (upper part). Representation of PVX-GFP, PVX-GFP/IV2 and PVX-GFP/IV2/ELVd(+) chimeric constructs (lower part). The constructs are not drawn to scale. B) GFP fluorescence stereomicroscope images of *N. benthamiana* leaves inoculated with PVX (unmodified), PVX-GFP, PVX-GFP/IV2 and PVX-GFP/IV2/ELVd(+) transcripts at 7 and 10 days post-inoculation. C) Serological detection of GFP. Total proteins were extracted from leaves, electrophoresed in 10% SDS-PAGE (upper panel) and blotted for serological detection (lower panel). GFP was clearly detected in both PVX-GFP and PVX-GFP/IV2/ELVd(+) systemically infected plants, confirming the correct processing of the chimeric GFP mRNA carrying the IV2/ELVd(+) insertion. D) RT-PCR amplification of processed and unprocessed GFP mRNAs. E) Confirmation (by RT-PCR) of systemic PVX infection in *N. benthamiana* plants inoculated with the construct used in this work.

Figure 2: Evidence for stability of chimeric constructs in systemically infected leaves. RT-PCR amplification of IV2 and ELVd(+) cDNAs imbibed in GFP open reading frame. The sequence and position of the specific primers (GFP-1 and 2 and IV2-1 and 2) used for amplification are detailed in Supplemental Figure 2. The sizes in the upper graph are not to scale.

Figure 3: Analysis of GFP expression in plants infected with PVX-derived transcripts carrying partial-length ELVd sequences. A) GFP fluorescence stereomicroscope images of *N. benthamiana* leaves inoculated with PVX-GFP, PVX-GFP/IV2/L-ELVd, PVX-GFP/IV2/R-ELVd and PVX-GFP/IV2/Up-ELVd transcripts at 6 and 9 and 12 days post-inoculation. B) Confirmation (by RT-PCR) of systemic PVX infection in *N. benthamiana* plants inoculated with the different constructs used in this assay. C) RT-PCR amplification of processed and unprocessed GFP mRNAs.

Figure 4: The full-length (plus strand) ELVd RNA mediates the trafficking of functional GFP-mRNA from the nucleus to chloroplasts. A) Physical map of the ELVd-5ᐭUTR-GFP and unmodified GFP constructs used in this assay. The construct sizes are not to scale. B) The ELVd-5ᐭUTR/GFP and GFP were clearly detected and showed similar relative electrophoretic mobility in the Western blot assay, indicating that the full-length viroid sequence acts as a true untranslated RNA. 1:5 serial dilutions are shown for each construct. C) Confocal microscope observation of *N. benthamiana* leaves expressing unmodified GFP (upper panels), ELVd-5ᐭUTR-GFP (central panels) or OE23/GFP (lower panels). The ELVd-5ᐭUTR/GFP mimics the cellular localization of the OE23/GFP construct, which accumulates specifically in chloroplasts.
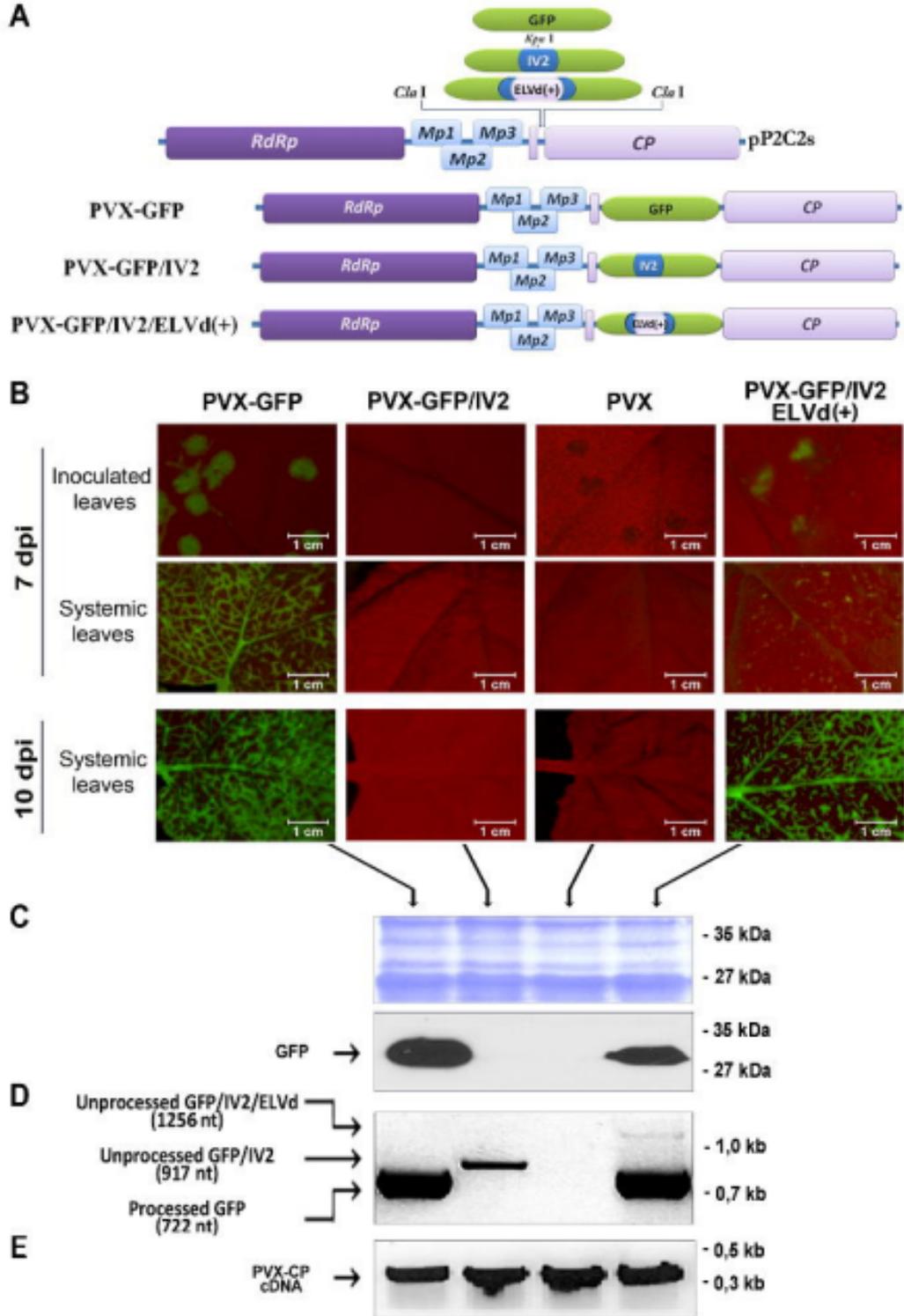
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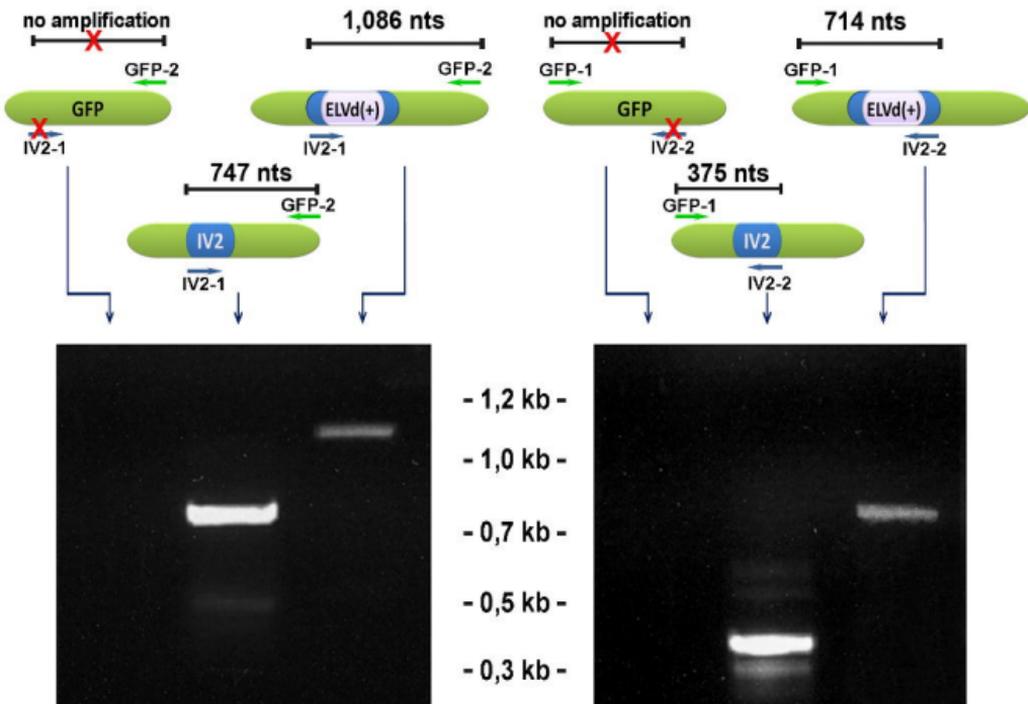
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Figure 3: Analysis of GFP expression in plants infected with PVX-derived transcripts carrying partial-length ELVd sequences. A) GFP fluorescence stereomicroscope images of N. benthamiana leaves inoculated with PVX-GFP, PVX-GFP/IV2-LELVd, PVX-GFP/IV2-R-ELVd and PVX-GFP/IV2Up-ELVd transcripts at 6 and 9 and 12 days post-inoculation. B) Confirmation (by RT-PCR) of systemic PVX infection in N. benthamiana plants inoculated with the different constructs used in this assay. C) RT-PCR amplification of processed and unprocessed GFP mRNAs.
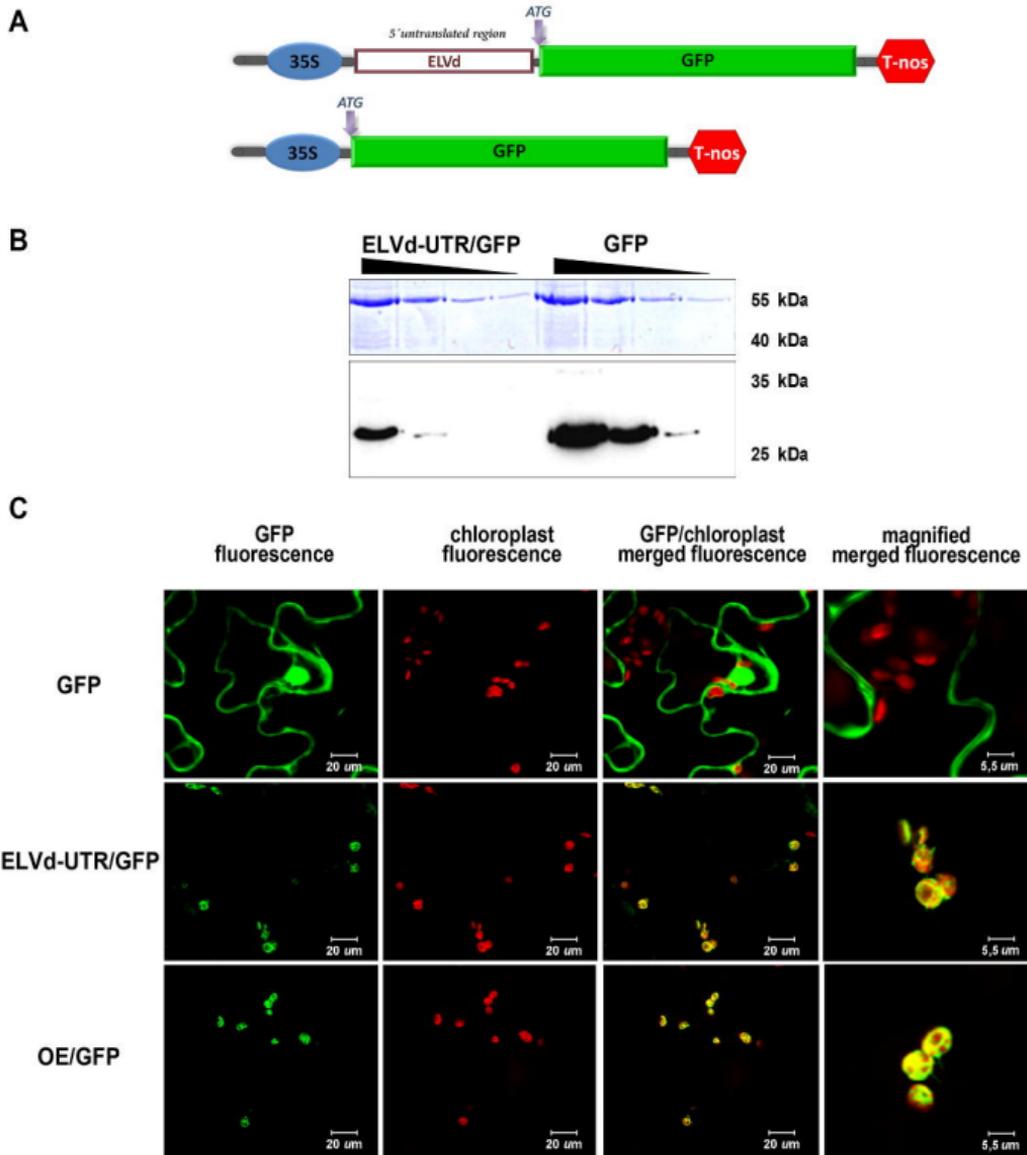
Figure 4: The full-length (plus strand) ELVd RNA mediates the trafficking of functional GFP-mRNA from the nucleus to chloroplasts. A) Physical map of the ELVd-5′UTR-GFP and unmodified GFP constructs used in this assay. The construct sizes are not to scale. B) The ELVd-5′UTR/GFP and GFP were clearly detected and showed similar relative electrophoretic mobility in the Western blot assay, indicating that the full-length viroid sequence acts as a true untranslated RNA. 1:5 serial dilutions are shown for each construct. C) Confocal microscope observation of N. benthamiana leaves expressing unmodified GFP (upper panels), ELVd-5′UTR-GFP (central panels) or OE23/GFP (lower panels). The ELVd-5′UTR/GFP mimics the cellular localization of the OE23/GFP construct, which accumulates specifically in chloroplasts.