University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Publications from USDA-ARS / UNL Faculty

U.S. Department of Agriculture: Agricultural
Research Service, Lincoln, Nebraska

2008

Nuclear targeting by fragmentation of the Potato spindle tuber viroid genome

Asta Abraitene
Institute of Biotechnology

Yan Zhao
zhaoy@ba.ars.usda.gov

Rosemarie Hammond

Follow this and additional works at: <https://digitalcommons.unl.edu/usdaarsfacpub>

Part of the [Agricultural Science Commons](#)

Abraitene, Asta; Zhao, Yan; and Hammond, Rosemarie, "Nuclear targeting by fragmentation of the Potato spindle tuber viroid genome" (2008). *Publications from USDA-ARS / UNL Faculty*. 389.
<https://digitalcommons.unl.edu/usdaarsfacpub/389>

This Article is brought to you for free and open access by the U.S. Department of Agriculture: Agricultural Research Service, Lincoln, Nebraska at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Publications from USDA-ARS / UNL Faculty by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Nuclear targeting by fragmentation of the *Potato spindle tuber viroid* genome

Asta Abraitienė^{a,b}, Yan Zhao^b, Rosemarie Hammond^{b,*}

^a Eukaryote Genetic Engineering Laboratory, Institute of Biotechnology, Vilnius, Lithuania

^b US Department of Agriculture, Agricultural Research Service, Molecular Plant Pathology Laboratory,
Room 214 Building 004 BARC-West, 10300 Baltimore Avenue, Beltsville, MD 20705, USA

Received 4 January 2008

Available online 22 January 2008

Abstract

Transient expression of engineered reporter RNAs encoding an intron-containing green fluorescent protein (GFP) from a *Potato virus X*-based expression vector previously demonstrated the nuclear targeting capability of the 359 nucleotide *Potato spindle tuber viroid* (PSTVd) RNA genome. To further delimit the putative nuclear-targeting signal, PSTVd subgenomic fragments were embedded within the intron, and recombinant reporter RNAs were inoculated onto *Nicotiana benthamiana* plants. Appearance of green fluorescence in leaf tissue inoculated with PSTVd-fragment-containing constructs indicated shuttling of the RNA into the nucleus by fragments as short as 80 nucleotides in length. Plant-to-plant variation in the timing of intron removal and subsequent GFP fluorescence was observed; however, earliest and most abundant GFP expression was obtained with constructs containing the conserved hairpin I palindrome structure and embedded upper central conserved region. Our results suggest that this conserved sequence and/or the stem-loop structure it forms is sufficient for import of PSTVd into the nucleus.

Published by Elsevier Inc.

Keywords: Viroid; Nucleus; *Potato virus X*; GFP; PSTVd; Hairpin I: central conserved region; Nuclear localization

Viroids are small, single-stranded, covalently-closed, circular RNA molecules that cause numerous diseases of plants [1]. Viroid genomes range in size from 245 to 401 nucleotides, depending upon the viroid species, and possess a host range that includes both herbaceous and woody plants [2]. There are currently 29 known viroid species that are grouped into two families, the *Pospiviroidae* (type member, *Potato spindle tuber viroid*, PSTVd) and the *Avsunviroidae* (type member, *Avocado sunblotch viroid*, ASBVd) based on sequence and structural homologies [2]. In general, members of the *Avsunviroidae* have a narrower host range than members of the *Pospiviroidae*. Unlike plant viruses, viroids lack mRNA activity and a protective protein capsid, and they do not require a helper virus for replication or movement [3]. As such, viroids rely upon interactions

with pre-existing host components for multiplication and spread throughout the plant, and, as these interactions are poorly understood, attempts to control viroid diseases by novel strategies have not been uniformly successful.

Key steps in the colonization of the host plant by a viroid are the ability of the viroid RNA to move systemically and its ability to move intracellularly from the cytoplasm to the subcellular compartment where viroid multiplication occurs. Systemic infection involves both the movement of viroid RNA from cell-to-cell through the plasmodesmata, and long distance movement paralleling the transport of photosynthate through the phloem. For *Potato spindle tuber viroid* (PSTVd), movement through the phloem involves the interaction with host proteins [4–6].

The subcellular compartment where viroid replication occurs differs between the two viroid families. Pospiviroids replicate in the nucleus and accumulate in the nucleolus [7,8], whereas avsunviroids have been shown to accumu-

* Corresponding author. Fax: +1 301 504 5449.

E-mail address: rose.hammond@ars.usda.gov (R. Hammond).

late, and presumably replicate, in the chloroplast [9,10]. Both employ a rolling-circle type of replication where linear, oligomeric RNA intermediates of both polarities (plus and minus) are synthesized using different host RNA polymerases. The intracellular movement of PSTVd into the nucleus from the cytoplasm was the focus of two recent studies. Woo et al. [11] found that nuclear transport of fluorescein-labelled PSTVd RNA in permeabilized tobacco protoplasts was mediated by a specific, saturable receptor and was cytoskeleton-independent and not coupled to the Ran GTPase cycle. In our studies using a whole plant assay, a full-length, 359 nt. PSTVd-embedded intron was inserted into the coding region of green fluorescent protein (GFP) [12]. When expressed in the cytoplasm, GFP expression was only seen for recombinant RNAs containing the PSTVd-containing intron, which provided a signal for nuclear import, allowing splicing to a functional RNA. Both of these studies suggested that the PSTVd molecule possesses a specific signal that directs it to the nucleus. The nature of that specific signal and the cellular factors involved are unknown.

Viroid molecules are presumed exist in their native state in the cell as rod-like structures, characterized by a series of short double helices and internal loops resulting from intramolecular base-pairing, as shown in Fig. 1 for PSTVd. The viroid molecule can be divided into five structural domains based primarily on sequence homologies between different viroids, but these regions also seem to be correlated with biological function [13]. In addition to the rod-like structure, thermal transitions of the viroid secondary structure occur upon melting of the molecule, with resulting formation of thermodynamically metastable structures [14] and transient secondary hairpins I, II, and III (Fig. 1) [15–17]. The hairpins, which are connected by single-stranded regions, are formed by long-range cooperativity and dissociation of the rod-like structure. PSTVd also contains a loop E motif in the central conserved region (Fig. 1). This motif, which is formed by interaction of the upper and lower strand, is involved in RNA–RNA and

RNA–protein interactions in a wide range of RNAs, and in PSTVd, the loop E region contains a processing domain for conversion of longer-than-unit length replicative intermediates into monomers [3].

It is not known if the entire PSTVd sequence is required for transit of the viroid into the nucleus or if portions of the RNA are sufficient. In this study, we dissected the PSTVd genome using the whole plant nuclear assay [12] to identify the core motifs that target PSTVd to the nucleus. Our results suggest that the evolutionarily-conserved HI – CCR sequence and/or the stem-loop structure it forms is sufficient to direct import of PSTVd into the nucleus.

Materials and methods

DNA constructions. The coding region of GFP was interrupted by insertion of an intron derived from the intervening sequence 2 (IV2) of the potato St-LS1 gene as described by Zhao et al. [12]. Fragments of PSTVd were amplified from viroid-containing plasmid DNAs and inserted into the intron as described below. The nomenclature of the constructs refers to their location on the rod-like, partially double-stranded structure of PSTVd (Fig. 1). All primers are listed in Table 1. The “upper half” of the intermediate strain of PSTVd (nt 1–180) was amplified using primers L1 and R1 from a head-to-tail dimer clone of PSTVd, pSTB14 [18], yielding L1R1. The lower half (nt 181–359; L2R2) was amplified using primers L2 and R2 from pSTB14. Hairpin I (HI) and the mutant HI (HIM) were constructed by using overlapping PCR and primer pairs HIL and HIR, or HIML and HIR, respectively. The primers contained either an SstI restriction or an XhoI restriction site at their 5' termini for subsequent cloning. The PCR products were cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA). Following restriction digestion, the amplified sequences were inserted into SstI and XhoI doubly-digested pUC19:IV2 [12]. Additional fragmentation of the PSTVd genome was performed by amplifying the top left (L1R3, nt 1–87) and top right (L3R1, nt 88–180) regions using primer pairs L1 and R3, and L3 plus R1, respectively (Fig. 1). The ASBVd sequence was amplified from a cDNA clone of ASBVd using primers ASBVF and ASBVR (Table 1). All subsequent subcloning of the fragment proceeded as above.

The PSTVd-containing intron constructs were digested with SnaBI and PvuII, and the fragments were cloned into the MscI restriction site of the *mGFP4* open reading frame as previously described [12]. The fragment-embedded, intron-bearing *gfp* reporter was amplified using the GFPF(Nru/EcoRV) and GFPR(Hind/Sal) primers (Table 1), and the

Fig. 1. Schematic drawing of the intermediate strain of PSTVd. The location of the five structural domains, secondary hairpins, loop E, and location of PSTVd fragments discussed in the text are indicated.

Table 1
Oligonucleotide primers used for plasmid constructions and RT-PCR analysis (shown 5′–3′)

Primer	Sequence
L1	GTTGGAGAGCTCCGGAACATAAAGTCGT
R1	GGAAGTCGAGAAACCCCTGTTTCGGC
L2	GTTGAGAGCTCCACCCTTCCTTTCTTC
R2	GGAAGTCGAGAGGAACCAACTGCGG
L3	CGCGAGCTCGGATCCCCGGGGAAACCTGGAGC
R3	GCGCTCGAGCTGAAGCGCTCCTCCGAGCC
HIL	CGCGAGCTCGGCGGCTCGGAGGAGCGCTTCAGGATCCCCGGGAAACCTGGAG
HIR	GCGCTCGAGCCGCTCTTTTGGCAGTTCGCTCCAGGTTTCCCGGG
HIML	CGCGAGCTCGGCGGCTCGGAGGAGCGgggCAGGATCCCCGGGAAACCTGGAG
ASBVF	CGCGAGCTCAAACTTTGTTTGACGAAACAGGTCTGTTCGACTTCCGACT
ASBVR	GCGCTCGAGCTCACAAGTCGAAACTCAGAGTCGGAAAGTCGG
GFPP(Nru/EcoRV)	GATCGCGATATCAACAATGTCTAAAGGAG
GFPR(Hind/Sal)	GCGTCGACAAGCTTAAAGGTATAGTTCA

Underlined nucleotides define restriction enzyme sites described in Materials and methods. Lower case letters in HIM designate the mutations introduced into the stem of Hairpin I.

amplicons were digested with EcoRV and HindIII, and cloned into an EcoRV + HindIII restricted intermediate PVX-fragment containing vector, pSKAS. pSKAS was previously constructed by cloning the the ApaI/SstI fragment of pP2C2S cloned into the pBluescript SK+ vector. An ApaI/SstI fragment containing the GFP reporter gene was excised from the pSKAS vector and was used to replace the corresponding ApaI/SstI fragment of the full-length PVX-based vector, pP2C2S. The resulting plasmid constructs were designated L1R1, L2R2, L1R3, L3R1, H1, and HIM, and correspond to their position on the PSTVd genome as shown in Fig. 1.

Transcription reactions and plant inoculations. The pP2C2S plasmid DNA constructs were linearized with SpeI prior to transcription. Approximately 1 µg of each linearized template was used in a 20 µl transcription reaction mix using the T7 mMessage mMachine kit (Ambion Inc., Austin, TX) at 37 °C to prepare capped transcripts. The reaction mixes were supplemented with 1 µl of 30 mM GTP 15 min after the start of the reaction to facilitate synthesis of long RNA transcripts. The transcription reactions were continued for an additional 90 min. An aliquot (1 µl) was analyzed on a 1.0% agarose gel to assess the quantity and quality of the RNA transcripts.

For plant inoculations, the transcripts were diluted to 0.5 µg/µl with 50 mM potassium phosphate (pH 7.0). Five microliters of the diluted transcripts were rubbed onto each of three carborundum-dusted leaves of *Nicotiana benthamiana* plants using a sterile glass rod. The plants were inoculated at the six-leaf stage and grown under greenhouse conditions under natural daylight with a temperature of 20–25 °C. The experiments were repeated at least three times.

Fluorescence detection. At 14 and 23 days post-inoculation, plants were examined using a FujiFilm Luminescent Image Analyzer LAS-1000plus (Fuji Photo Film Co., LTP, Tokyo, Japan). The images were obtained using LAS-1000 Pro software and colorized using Adobe Photoshop Version 7.0 Software.

Nucleic acid extractions and reverse transcription-polymerase chain reaction (RT-PCR) assays. Total nucleic acids were extracted from systemically-infected leaf tissue 2 weeks after inoculation using TRI Reagent (Molecular Research Inc., Cincinnati, OH). Two microgram of total nucleic acid was amplified using 20 pmol of each primer [GFPP(Nru/EcoRV)/GFPR(Hind/Sal), Table 1] and 200 µM dNTPs in a 50 µl reaction of the Titan One-tube RT-PCR kit (Roche Molecular Biochemicals, Bedford, MA). The amplification conditions were 5 min at 95 °C, and 35 cycles of 1 min at 95 °C, 2 min 42 °C, 3 min 68 °C, followed by 7 min 68 °C. The amplicons were electrophoresed through 1% agarose gels containing 1× TBE buffer. RT-PCR controls contained nucleic acid from uninoculated *N. benthamiana* plants. Size controls included RT-PCRs using the same primers and amplification conditions on the plasmid DNA templates used for transcription. The amplicons were sequenced using the GFPP (Nru/EcoRV) and GFPR (Hind/Sal) primers.

Results and discussion

Characterization of GFP fluorescence

The appearance of green fluorescence in leaf tissue was monitored after inoculation with each fragment-containing construct (Fig. 2). There was no fluorescence in the uninoculated (Healthy), or in GFPint (12; negative control), and ASBVd construct containing plants. ASBVd replicates in the chloroplast and does not appear to enter the nucleus, demonstrating the specificity of this assay (Fig. 2). There was little fluorescence from the L2R2 construct that represents the lower portion of the viroid molecule, whereas the number and appearance of fluorescent lesions was very stable with the full-length PSTVd insertion (PVX-GFPintPSTVd) and L1R1 (the upper strand of PSTVd) (Fig. 2). As the L1R1 construct retained its ability to target the viroid to the nucleus, further dissection of this region was performed. Both L1R3 and L3R1 exhibited fluorescence, but the highest plant-to-plant variability was observed with the L3R1 fragment (upper left region), with the appearance of lesions varying from 1 to 3 weeks post-inoculation. The highly structured HI caused the earliest and most abundant production of functional GFP. Expression of fluorescence was not interrupted or significantly affected by the three mutations (CUU → GGG) that are predicted to disrupt the base-pairing in the hairpin stem (HIM, Fig. 2).

RT-PCR assays of intron splicing

Intron removal from each construct was confirmed by RT-PCR using primers that flank the GFP reporter gene. The L1R1 construct, which exhibited high levels of fluorescence, resulted in efficient excision of the intron from GFP and the production the predicted RT-PCR product of 740 bp (Fig. 3A, lane 4), whereas the L2R2 construct with low fluorescence, resulted in partial excision products, with approximately 40% completely unspliced product (Fig. 3A,

Fig. 2. Demonstration of nuclear targeting by GFP fluorescence. Representative plants from transcript inoculations containing the various GFP constructs examined at 14 days post-inoculation. Negative controls: uninoculated *N. benthamiana* (Healthy), PVX-GFP intron (PVX-GFPint) ASBVd (ASBVd); Positive controls, PVX-GFP and PVX-GFPintPSTVd, containing the full-length PSTVd sequence. L1R1 and L2R2 are the 'top' and 'bottom' of PSTVd, and L1R3 (salmon), L3R1 (blue), H1 (yellow), and HIM (black) are the remaining fragments. The location of the mutations in HIM is boxed.

lane 2). GFP containing the intron alone did not fluoresce (Fig. 2) and the intron was not excised (Fig. 3A, lane 5).

The RT-PCR assay also revealed that there was plant-to-plant and construct-dependent variation in splicing, as the intron was completely removed in some plants, and not removed in others (Fig. 3B). For example, the L1R1 construct was mostly spliced in all plants, but there was plant-to-plant variation in L1R3 and L3R1. The H1 and HIM constructs were efficiently spliced, as well as the complete PSTVd construct, while ASBVd-containing construct was not spliced (Fig. 3B, lane 19). These results confirm what was found in the fluorescence assay (Fig. 2). Selected

amplicons were sequenced or restricted as described [12] and the results demonstrate the excision of the intron (data not shown).

In summary, our experimental evidence shows that H1 was able to shuttle a non-viroid RNA into the nucleus and that this function was not interrupted by three mutations which were predicted to disrupt the base-pairing in the stem of the hairpin. However, even when H1 was split (fragments L1R3 and L3R1 of 87 and 93 nt in length, respectively), the viroid sequences were still able to shuttle the RNA into the nucleus. These constructs were more efficient at targeting than the full-length viroid of 359 nt or the

Fig. 3. Evidence for RNA splicing by characterization of intron and PSTVd-fragment bearing GFP constructs using an RT-PCR assays and flanking GFP primers. (A) Characterization of splicing activities of L1R1 and L2R2 variants. Lane 1, uninoculated *N. benthamiana*; Lane 2, L2R2; Lane 3, GFP; Lane 4, L1R1; Lane 5, GFPIV. Lane M, molecular size standards are Lambda DNA digested with EcoRI + HindIII (Promega Inc.). Mobility standards for splicing are GFP (c₁, 740 bp), GFP/IV (c₂, 940 bp), GFP/IV/L1R1 (c₃, 1120 bp), and GFP/IV/PSTVd (c₄, 1300 bp), respectively. (B) Variant efficiency of RNA splicing obtained by RT-PCR assay using total plant RNA extracted 2 weeks post inoculation. Lanes M, c₁, c₂, c₃, and c₄ as for 3A; remaining lanes represent individual *N. benthamiana* plants inoculated with PSTVd-fragment containing constructs. Lanes 1, 2, 10, 11—HI; lanes 3, 4, 12—HIM; lanes 5, 6, 7—L1R1; lanes 8, 9, 15, 16—L1R3; lanes 13, 14—L3R1; Lanes 19, 20, 21—ASBVd; lanes 17 and 18—PSTVd.

lower half of the molecule (L2R2), but were less efficient than the HI and HIM hairpin constructs. The appearance of more or less GFP fluorescence and processing in each construct suggests the possibility of more than one nuclear localization signal or motif in the upper portion of the PSTVd genome (nt 1–180). Alternatively, the splicing efficiency may be influenced by the intron/exon boundaries [19]. However, HI alone can function in movement of PSTVd-containing RNAs into the nucleus.

The conservation of HI and the upper CCR indicates their importance in the life cycle of pospiviroids. The CCR is known to contain the structural and functional requirements for correct processing of longer-than-unit length replicative intermediates [3]. Mutations in HI that disrupted the stem of the hairpin abolished infectivity [20]. The conserved GAAA tetraloop (loop E) is essential for processing by favoring a kinetically controlled conformation essential for ligation. HII, with its GC-rich stem, includes most of the lower strand in the rod-like PSTVd structure (Fig. 1), and has been shown to play an important role in viroid replication [17]. The lower portion of the viroid (L2R2) did not shuttle the RNA into the nucleus, therefore, it most likely does not play a role in nuclear localization.

The translocation of cellular factors across the nuclear envelope is a controlled process [21]. With the nuclear pore diameter of 10 nm and a 60–70,000 Da diffusion limit, a viroid RNA molecule in its renatured state of 37 ± 6 nm, ~100,000 Da [15] would not diffuse into the nucleus. Other RNAs which transit the nuclear membrane do so with the assistance of proteins, and multiple pathways may be used, e.g., snRNAs possess a cap structure and core proteins, tRNAs actively shuttle between the nucleus and cytoplasm using an energy-dependent process and interaction with exportin-t [22], mRNAs possess a cap structure and interact with hnRNP proteins. The snoRNA domain of the vertebrate telomerase RNA functions in nuclear retention and nucleolar localization; this domain contains a box H/ACA motif [23]. Recently, it has been shown that a 6 nucleotide, 3' terminal sequence element (AGUGUU) of a specific micro RNA (miR-29b), could function as a transferable nuclear localization signal when attached to the 3' terminus of other 22–21 nucleotide micro RNAs [24]. The HI of PSTVd does not contain the specific AGUGUU motif; however, it demonstrates that nuclear localization signals are also transferable in other systems.

Viroid nuclear import may result from the formation of a nucleoprotein complex with cellular factors. PSTV is

known to interact with host proteins, including histones, an S25 ribosomal protein, the large subunit of tomato DNA-dependent RNA polymerase I, and phloem protein-2, among others [3]. Recently, a tomato bromodomain-containing protein, VirpI, was shown to bind specifically to motifs in the right terminal domain of PSTVd both *in vitro* and *in vivo* [25]. VirpI is member of a family of transcriptional regulators that are associated with chromatin remodeling. In addition to the bromodomain motif, VirpI contains a putative nuclear localization signal and was proposed to be a candidate to mediate nuclear transfer of the viroid into and out of the nucleus. Although, we do not have evidence that disruption of the VirpI-binding sites lead to loss of nuclear shuttling, our results suggest that the right terminal domain is not the primary nuclear localization signal.

Many questions still remain to be answered. Does the same sequence/structural motif also determine directional traffic of PSTVd into the nucleolus or out of the nucleus? It has recently been shown that the plus strand of PSTVd localizes to the nucleoplasm, where the negative strand localizes to the nucleolus as well as the nucleoplasm [26]. Does the viroid attach to nuclear pore proteins itself or does it interact with cytoplasmic proteins that facilitate its movement into the nucleus? Our finding that a sequence element may be functional without the complete viroid sequence may allow isolation of nuclear pore proteins or other host proteins that interact with the RNA and help to delineate the basic nuclear transport machinery of plant cells. An additional application of this knowledge would be the disruption of the viroid–host interaction(s) leading to nuclear import and, perhaps, subsequent disease control.

Acknowledgments

We acknowledge the assistance of Dr. Rokas Abraitis, Dr. Nancy Kreger, Sharon Jhingory, and Emily Kuykendall who participated in various aspects of the project.

References

- [1] T.O. Diener, Potato spindle tuber virus. IV. A replicating, low molecular weight RNA, *Virology* 45 (1971) 411–428.
- [2] A. Hadidi, R. Flores, J.W. Randles, J.S. Semancik (Eds.), *Viroids*, CSIRO Publishing, Australia, 2003.
- [3] M. Tabler, M. Tsagris, *Viroids: petite RNA pathogens with distinguished talents*, *Trends Plant Sci.* 9 (2004) 339–348.
- [4] G. Gomez, V. Pallas, Identification of an *in vitro* ribonucleoprotein complex between a viroid RNA and a phloem protein from cucumber plants, *Mol. Plant Microbe Interact.* 14 (2001) 910–913.
- [5] R.A. Owens, M. Blackburn, B. Ding, Possible involvement of the phloem lectin in long-distance viroid movement, *Mol. Plant Microbe Interact.* 14 (2001) 905–909.
- [6] Y. Zhu, Y. Qi, Y. Xun, R. Owens, B. Ding, Movement of potato spindle tuber viroid reveals regulatory points of phloem-mediated RNA traffic, *Plant Physiol.* 130 (2002) 138–146.
- [7] J. Harders, N. Lukacs, M. Robert-Nicoud, J.M. Jovin, D. Riesner, Imaging of viroids in nuclei from tomato leaf tissue by *in situ* hybridization and confocal laser scanning microscopy, *EMBO J.* 8 (1989) 3941–3949.
- [8] I.M. Schindler, H.P. Mühlbach, Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: a reevaluation, *Plant Sci.* 84 (1992) 221–229.
- [9] R.G. Bonfiglioli, G.I. McFadden, R.H. Symons, *In situ* hybridization localizes avocado sunblotch viroid on chloroplast thylakoid membranes and coconut cadang cadang viroid in the nucleus, *Plant J.* 6 (1994) 99–103.
- [10] J.A. Navarro, A. Vera, R. Flores, A chloroplast RNA polymerase resistant to tagetitoxin is involved in replication of avocado sunblotch viroid, *Virology* 268 (2000) 218–225.
- [11] Y.-M. Woo, A. Itaya, R.A. Owens, L. Tang, R.W. Hammond, H.-C. Chou, M.M.C. Lai, B. Ding, Characterization of nuclear import of potato spindle tuber viroid RNA in permeabilized protoplasts, *Plant J.* 17 (1999) 627–635.
- [12] Y. Zhao, R.A. Owens, R.W. Hammond, 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 (2001) 1491–1497.
- [13] P. Keese, R.H. Symons, Domains in viroids: evidence of intermolecular rearrangements and their contribution to viroid evolution, *Proc. Natl. Acad. Sci. USA.* 82 (1985) 4582–4586.
- [14] D. Repsilber, S. Wiese, M. Rachen, A. Schröder, D. Riesner, G. Steger, Formation of metastable RNA structures by sequential folding during transcription: time-resolved structural analysis of potato spindle tuber viroid (–)-stranded RNA by temperature-gradient gel electrophoresis, *RNA* 5 (1999) 574–584.
- [15] K. Henco, H.L. Sängler, D. Riesner, Fine structure melting of viroids as studied by kinetic methods, *Nucleic Acids Res.* 6 (1979) 3041–3059.
- [16] D. Riesner, K. Henco, U. Rokohl, G. Klotz, A.K. Kleinschmidt, H. Domdey, P. Jank, H.J. Gross, H.L. Sängler, Structure and structure formation of viroids, *J. Mol. Biol.* 133 (1979) 85–115.
- [17] A.R.W. Schröder, D. Riesner, Detection and analysis of hairpin II, an essential metastable structural element in viroid replication intermediates, *Nucleic Acids Res.* 30 (2002) 3349–3359.
- [18] R.W. Hammond, T.O. Diener, R.A. Owens, Infectivity of chimeric viroid transcripts reveals the presence of alternative processing sites in potato spindle tuber viroid, *Virology* 170 (1989) 486–495.
- [19] D. Lewandowska, C.G. Simpson, G.P. Clark, N.S. Jennings, M. Barciszewska-Pacak, C.F. Lin, W. Makalowski, J.W.S. Brown, A. Jarmolowski, Determinants of plant U-12-dependent intron splicing efficiency, *Plant Cell* 16 (2004) 1340–1352.
- [20] R.W. Hammond, R.A. Owens, Mutational analysis of potato spindle tuber viroid reveals complex relationships between structure and infectivity, *Proc. Natl. Acad. Sci. USA* 84 (1987) 3967–3971.
- [21] L.J. Terry, E.B. Shows, S.R. Wente, Crossing the nuclear envelope: hierarchical regulation of nucleocytoplasmic transport, *Science* 318 (2007) 1412–1416.
- [22] A. Takano, T. Endo, T. Yoshihisa, tRNA actively shuttles between the nucleus and cytosol in yeast, *Science* 309 (2005) 140–142.
- [23] A.A. Lukowiak, A. Narayanan, Z.-H. Li, R.M. Terns, M.P. Terns, The snoRNA domain of vertebrate telomerase RNA functions to localize the RNA within the nucleus, *RNA* 7 (2001) 1833–1844.
- [24] H.-W. Huang, E.A. Wentzel, J.T. Mendell, A hexanucleotide element directs microRNA nuclear import, *Science* 315 (2007) 97–100.
- [25] M. Gozmanova, M.A. Denti, I.N. Minkov, M. Tsagris, M. Tabler, Characterization of the RNA motif responsible for the specific interaction of potato spindle tuber viroid RNA (PSTVd) and the tomato protein VirpI, *Nucleic Acids Res.* 31 (2003) 5534–5543.
- [26] Y. Qi, B. Ding, Differential subnuclear localization of RNA strands of opposite polarity derived from an autonomously replicating viroid, *Plant Cell* 15 (2003) 2566–2577.