Use of a vector based on *Potato virus X* in a whole plant assay to demonstrate nuclear targeting of *Potato spindle tuber viroid*

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*Potato spindle tuber viroid* (PSTVd) is a covalently closed circular RNA molecule of 359 nucleotides that replicates within the nucleus of host cells. To determine how this small, highly structured RNA enters the nucleus, we have developed a virus-based, whole plant *in vivo* assay that uses green fluorescent protein (GFP) as the reporter molecule. The coding region of GFP was interrupted by insertion of an intron derived from the intervening sequence 2 of the potato ST-LS1 gene. A cDNA copy of the complete PSTVd genome was, in turn, embedded within the intron, and this construct was delivered into *Nicotiana benthamiana* plants via a vector based on *Potato virus X*. The intron-containing GFP subgenomic RNA synthesized during virus infection cannot produce a functional GFP unless the RNA is imported into the nucleus, where the intron can be removed and the spliced RNA returned to the cytoplasm. The appearance of green fluorescence in leaf tissues inoculated with constructs containing a full-length PSTVd molecule embedded in the intron indicates that nuclear import and RNA splicing events did occur.

Introduction

Viroids are the smallest infectious agents known. Covalently closed, circular RNA molecules ranging in size from 250 to 450 nucleotides, viroids replicate and move from cell to cell without a helper virus and are located and replicated in the nucleus (or chloroplast in some cases) (Bonfiglioli et al., 1994, 1996; Harders et al., 1989; Navarro et al., 1999; Schindler & Mühlbach, 1992). Unlike viruses, viroids are not encapsidated and do not appear to encode any proteins. Therefore, their replication and movement must rely on direct interactions between the viroid genome and unidentified host factors (Dieder, 1987). The nature and the underlying mechanisms of these interactions are unknown.

The establishment of a systemic virus or viroid infection in a susceptible host depends on the ability of the pathogen to replicate and spread throughout that host. This process involves both intra- and intercellular transport of viral and viroid nucleic acids. Studies of human and animal, as well as plant, viruses have shown that, for viruses which replicate in the nucleus, virus-encoded protein(s) play important roles in shuttling the viral genome to and from its site of replication (Bukrinskaya et al., 1996; Nakanishi et al., 1996; Chou et al., 1998; Whittaker & Helenius, 1998). Many of the viral proteins involved have been shown to contain nuclear localization signals (Schmolke et al., 1995; Ye et al., 1995; Watanabe et al., 1996; D’Agostino et al., 1997; Chou et al., 1998) and other regulatory motifs (Seydel & Jans 1996; Xiao et al., 1996; Bichko et al., 1997; Kubota & Pomerantz, 1998) that can interact with host factors involved in the nuclear transport pathway (Gorlich & Mattaj, 1996; Greber et al., 1997). In the case of the geminivirus *Squash leaf curl virus*, interaction of virus-encoded proteins BR1 and BL1 is required for movement of the viral genome from cell-to-cell, but only BR1 functions in nuclear import (and export) of the viral genome (Sanderfoot & Lazarowitz, 1995; Sanderfoot et al., 1996; Ward & Lazarowitz, 1999). BR1 contains domain structures required for nuclear targeting and interaction with BL1, and a nuclear export sequence has been identified in BR1 (Sanderfoot et al., 1996; Ward & Lazarowitz, 1999). In another geminivirus, *Maize streak virus*, it has been shown that the viral coat protein facilitates the transfer of viral DNA into the nucleus (Liu et al., 1999). In the nucleorhabdovirus *Sonchus yellow net virus*, which also replicates in the nucleus, the nucleocapsid protein and the phosphoprotein (M2) are primarily localized to the nucleus, although their role in nuclear transport of the viral RNA is unknown (Martins et al., 1998). Much less is known about the possible role of RNA or DNA sequence motifs in nuclear transport.
Recently, Woo et al. (1999) examined the translocation of fluorescein-labelled Pot<sub>ato</sub> spindle tuber viroid (PSTVd) to the nuclei in permeabilized tobacco cells. The results of their study indicate that PSTVd import may be a cytoskeleton-independent process that is mediated by a specific and saturable receptor. Previous efforts to use plant virus-based episomal vectors to produce useful products or to study virus movement, gene regulation and host–pathogen interactions have focused on events occurring in the host cell cytoplasm (see Donson et al., 1991; Sablowski et al., 1995; Oparka et al., 1996). Here, we describe an experimental system that utilizes a vector derived from the plant cytoplasmic virus <em>Potato virus X</em> (PVX) to study and ultimately identify the nuclear targeting signals of PSTVd in a whole plant system.

**Methods**

- **Viroid cDNA, the GFP reporter and the virus-based vector.** A full-length cDNA copy of the intermediate strain of PSTVd was excised at the <em>Bam</em>H<sub>II</sub> sites from the plasmid vector pSP64 (Owens et al., 1986). A modified jellyfish green fluorescent protein (GFP) gene, mgfp<sub>4</sub>, was obtained from Jim Haseloff (Cambridge, UK). In mgfp<sub>4</sub>, the codon usage of the original <em>gfp</em> has been modified to avoid a cryptic intron (Haseloff et al., 1997). The PVX-based vector, pP2C2S, was a gift from David Baulcombe of the Sainsbury Centre, UK.

- **Construction of the intron-bearing reporter genes.** The coding region of GFP was artificially interrupted by inserting an intron derived from intervening sequence 2 (IV<sub>2</sub>) of the potato ST-LS1 gene. The second intron of the potato ST-LS1 gene (Eckes et al., 1986; GenBank accession no. X04401) was amplified by PCR and modified with a strategy similar to that described by Vancanneyt et al. (1990). The modifications made to the intron include: (i) addition of a <em>Bam</em>H<sub>II</sub> recognition site to a position in the middle of the intron; (ii) alteration of the border sequences to introduce a SnaBI and a P<sub>oul</sub>ll site at the 5′ and 3′ termini, respectively; and (iii) optimization of internal intron sequences with respect to the consensus sequence for plant introns (see Fig. 2). At this point, the intron was digested with SnaBI and P<sub>oul</sub>ll and inserted into an <em>Msi</em>I site of mgfp<sub>4</sub> within the GFP open reading frame. A full-length (359 nucleotide) copy of the intermediate strain of PSTVd was then embedded in the intron at the <em>Bam</em>H<sub>II</sub> site, in the ‘positive-sense’ orientation from nucleotide 88 to nucleotide 87 of PSTVd. This intron-bearing and PSTVd-embedded <em>gfp</em> reporter gene was cloned into the SnaI site of the PVX-based vector pP2C2S.

- **Preparation and delivery of infectious transcripts.** The PVX-based vector pP2C2S contains a bacteriophage T7 promoter upstream from the respective viral cDNA template, which facilitates the production of infectious viral transcripts <em>in vitro</em> using T7 RNA polymerase-driven transcription. The plasmid DNAs pPVXGFP, pPVXGFPIV<sub>2</sub> and pPVXGFPIV<sub>2</sub>PSTVd were linearized with SpeI prior to transcription. Approximately 1 μg of each DNA template was used in a 20 μl reaction mixture for synthesis of capped transcripts using a T7 mMessage mMachine kit (Ambion) at 37 °C. Because of the size of the expected transcripts, the reaction mix was supplemented with 1 μl of 30 mM GTP 15 min after the start of the reaction. The transcription reaction was continued for 90 min. An aliquot of the product (2 μl) was analysed on a 1% agarose gel to assess the quantity and the integrity of the transcripts.

For inoculation onto plant hosts, the transcripts were diluted to 0.5 μg/μl with 50 mM potassium phosphate (pH 7.0). Five μl of the diluted transcripts was gently rubbed onto each carborundum-dusted leaf of <em>Nicotiana benthamiana</em> plants using a sterile glass rod. The largest leaf of the three inoculated leaves was approximately 5 cm in length.

- **Microscopic analysis of the plants.** Five to 15 days after inoculation, the inoculated and systemically infected leaves of <em>N. benthamiana</em> were examined with a Zeiss epifluorescence microscope (Axioskop 2) for the presence of green fluorescence. Green fluorescence was detected using an FITC multiband filter set (#41001, Chroma) with a blue excitation filter (HQ480/40), a dichroic mirror (Q505LP) and a green barrier filter (HQ535/50). Photographs were taken with Kodak Ektachrome Elite II film 400 using the microscope-coupled SLR camera and automatic exposure meter.

- **Reverse transcriptase–PCR.** Total cellular RNA was prepared from the inoculated and systemic <em>N. benthamiana</em> leaves 2 weeks after inoculation of the PVX constructs using TRI REAGENT (Molecular Research Center). Five μg of the total RNA was incubated at 65 °C for 4 min before being reverse transcribed at 42 °C for 90 min using 400 units of M-MLV reverse transcriptase (Life Technologies) in a 25 μl reaction mixture containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 1 mM dNTPs, 20 pmol of GFP reverse primer (5′ GATTCCTGATCGACCTTTAAGGTATAGTTT 3′), 20 units RNasin (Promega) and 1 mM DTT. Two μl of the resulting first strand cDNA was heated at 94 °C for 5 min and amplified by the GFP forward (5′ GGATACCGGTCGACATAACAATGTCTAAAGG 3′) and reverse primer pair in a 100 μl reaction mixture containing 1 × Perkin Elmer PCR buffer I, 200 μM dNTPs, 2.5 units AmpliTaq DNA polymerase (Perkin Elmer, Roche Research Systems) and 50 pmol of each primer. The amplification conditions were 1 min at 94 °C for denaturation, 2 min at 42 °C for annealing and 2 min at 72 °C for synthesis. The PCR fragments were fractionated on a 1% agarose gel. RT–PCR controls included nucleic acid obtained from a healthy <em>N. benthamiana</em> plant and from a plant infected with the PVX vector lacking the GFP constructs.

**Results**

To establish a system for identification of specific sequence elements that are required for RNA movement into the host cell nucleus, a modified version of the GFP, mgfp<sub>4</sub>, was used as a reporter molecule. In mgfp<sub>4</sub>, the codon usage of the original <em>gfp</em> gene was modified to avoid a cryptic intron (Haseloff et al., 1997). The coding region of GFP was interrupted by inserting an intron, IV<sub>2</sub>, of the potato ST-LS1 gene. A full-length PSTVd sequence was in turn embedded in the intron, and the resultant intron and PSTVd-bearing GFP reporter genes were placed behind the subgenomic promoter of a PVX-based vector (pP2C2S) (Fig. 1). The constructs, once transcribed <em>in vitro</em> with T7 RNA polymerase, take the form of infectious PVX genomic RNA. Upon inoculation onto leaves of susceptible plant hosts, the virus will replicate in the infected host cells, and the intron-bearing reporter gene will be expressed in the cytoplasm as a subgenomic RNA (messenger RNA) of the viral genome.

As shown in Fig. 2(B), green fluorescence was readily observable during a period of 5 to 15 days after inoculation in leaf tissues from the plants inoculated with the intron-bearing and PSTVd genomic strand-embedded PVX construct, indicating the functional expression of the GFP. While the fluorescence is readily observable in mesophyll as well as
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Fig. 1. Construction of the intron-bearing gfp reporter gene shuttled by a PVX-based vector. (A) PVX-based vector pP2C2S. (B) The open reading frame of the jellyfish green fluorescent protein gene, mgfp4, was interrupted by inserting an artificial intron at the MscI site; a full-length copy of the PSTVd cDNA was in turn embedded into the intron at the BamHI site in ‘positive-sense’ orientation. (C) DNA sequences around the artificial exon/intron junctions. RdRp, RNA-dependent RNA replicase; M1, M2, M3, triple gene block movement proteins; CP, capsid protein.

Fig. 2. In planta GFP expression from the intron- and PSTVd-bearing virus construct. Fifteen days post-inoculation of N. benthamiana plants, whole systemically infected leaves were examined for green fluorescence. Photographs were taken with an epifluorescence microscope equipped with a multiband FITC filter set. (A) Positive control inoculated with PVXGFP; (B) sample inoculated with PVXGFPIV2,PSTVd; and (C) negative control inoculated with PVXGFPIV2. Bars on the micrographs represent 200 µm.

epidermal cells, it tends to be more intense in vascular tissues (data not shown). Since PVX replicates exclusively in the cytoplasm, the cytoplasmically localized intron-bearing messenger RNA will not produce a functional GFP unless (i) the subgenomic RNA is targeted to the nucleus, where the intron can be precisely removed, and (ii) the spliced mRNA is returned to the cytoplasm and translated. The presence of green fluorescence in epidermal cells inoculated with the PSTVd-embedded intron-bearing gfp construct implied that the above-stated nuclear trafficking events had occurred. In the same experiment, a negative control construct, GFPIV2 (which had no PSTVd sequence embedded in the intron), did not produce any observable green fluorescence (Fig. 2C), indicating that an mRNA that could be translated into a functional GFP protein in the cytoplasm was not produced.

Expression of a functional protein from an intron-containing gene is strong circumstantial evidence of proper mRNA processing, which depends on the functions of the nuclear-
Fig. 3. Evidence for RNA splicing. (A) Characterization of the intron- and PSTVd-bearing gfp RNA as revealed by RT–PCR using mgfp4 primers. Lane M, molecular size standards; lanes 1–3, samples from leaves of an N. benthamiana plant inoculated with PVXGFP; lanes 4–9, samples from leaves of two representative N. benthamiana plants inoculated with PVXGFP,VPSTVd; lanes 10 and 11, negative controls of RT–PCR. Lane 10 is a sample from a leaf of healthy N. benthamiana; lane 11 is a sample obtained from a plant inoculated with the PVX vector; lanes 12 and 13 show mobility standards for GFP and GFPIV2PSTVd (GIV), respectively. (B) Restriction analysis of the RT–PCR amplified reporter molecules. Lane M, molecular size standards; lane 1, MscI digestion of the RT–PCR product shown in (A, lane 1) (GFP); lane 2, MscI digestion of the RT–PCR product shown in (A, lane 7) (GIV); lanes 3 and 4, mobility standards for MscI digestion of GFP and GIV, respectively; lanes 5 and 6, mobility standards for intact GFP and GIV, respectively.

dwelling splicing apparatus. To obtain further evidence that the RNA splicing event did indeed occur, the messenger RNA for the intron/PSTVd-bearing gfp reporter gene was amplified by RT–PCR and mapped with the restriction enzyme MscI.

Fig. 3(A) shows the expression profiles of the intron/PSTVd-bearing gfp gene in leaves of N. benthamiana plants sampled 2 weeks post-inoculation. A band of 740 bp corresponding to the spliced GFP open reading frame was detected in all sample leaves in which the intron/PSTVd-bearing gfp gene was expressed (lanes 4, 6, 7, 8, 9), and in those leaves sampled from plants inoculated with the gfp gene lacking the intron (lanes 1–3). Lane 5 corresponds to a leaf that had not yet become infected. In addition to the correctly spliced product, some samples containing the intron/PSTVd-bearing gfp gene contained residual, unspliced RNA of 1300 bp (e.g. lanes 7 and 9). The accurate removal of the intron was confirmed by the restoration of the MscI site in the GFP open reading frame (Fig. 3B, lane 2), as evidenced by restriction analysis of RT–PCR products yielding bands of 548 and 192 bp. Again, residual, unspliced RNA is present in one of the samples (1300 bp, lane 2). The presence of unspliced RNA in several of the samples indicates that either import of the viroid-containing RNA to the nucleus and/or splicing of the intron-containing RNA and return to the cytoplasm are not 100% efficient.

To address the possibility that, in the parent virus, the IV,PSTVd sequence was spliced from the genomic sequence, thereby yielding a functional GFP protein, PVX virions were
isolated from fluorescent, systemically infected leaves of *N. benthamiana* plants 3 weeks after pPVXGFPV2_PSTVd transcript inoculation (Hammond & Lawson, 1988). Viral genomic RNA was purified from the virions using TRI REAGENT and was subjected to RT–PCR analysis using the GFP forward and reverse primers (Fig. 4). The GFPIV2_PSTVd construct is stable at the genomic RNA level, as revealed by the presence of the unspliced 1300 bp PCR product in lane 1.

**Discussion**

We have described an experimental system that employs a cytoplasmic virus vector to study nuclear import of RNA molecules. The fact that addition of the PSTVd sequence to an intron-containing reporter mRNA leads to the expression of a functional GFP suggests that the viroid possesses cis-acting signal(s) for nuclear targeting of RNA. Without such signal(s), the intron-bearing GFP mRNAs expressed in the cytoplasm cannot be processed to form a complete GFP open reading frame since the RNA splicing apparatus resides exclusively in the nucleus. The nature of this putative targeting signal remains to be determined, however.

In nature, PSTVd is a circular molecule that assumes an unbranched rod-like structure through intramolecular base pairing. However, in our experimental system, this circular molecule was artificially linearized and attached to a recombinant PVX subgenomic RNA of more than 1 kb, yet the nuclear-targeting signal(s) remained functional in this chimeric molecule. In this regard, it is of particular interest to note that both the ability of PSTVd to move from cell-to-cell (Ding et al., 1997) and its ability to act as a promoter for a viral RNA replicase (Maroon, 1997) are preserved when the PSTVd molecule is linearized and fused to non-viroid sequences. Likewise, our data indicate that the nuclear targeting capacity of the viroid is retained in the linear form, and, with the reporter system, the nucleotide sequence motifs required for nuclear-targeting should be identifiable.

The ability of linearized and tagged PSTVd to retain its nuclear-targeting functions does not necessarily mean that the nuclear-targeting motif(s) resides in the linear (or primary) sequence of the viroid. Theoretical calculations using the mfold program (Wisconsin package version 9.1, 1997; Zuker, 1989) indicate that the PSTVd sequences embedded within the intron are a distinct domain that retains a rod-like, i.e. `native' conformation (data not shown). For PSTVd and related viroids, the rod-like skeleton can be divided into five structural and functional domains (Keese & Symons, 1985) and there is a strong correlation between various biological properties and individual domain structures (Hammond, 1992; Hammond & Owens, 1987; Owens et al., 1986, 1995, 1996; Sano et al., 1992). These individual domain structures will be examined in our future experiments to locate the precise nuclear-targeting motif(s).

The mechanisms of recognition of viroid nuclear localization signals by putative host factors remain to be elucidated. Being a circular, rod-like, nuclear-replicating RNA, human Hepatitis delta virus (HDV) shares many common features with viroids; its single open reading frame encodes two amino-coterminal proteins known as hepatitis delta antigen (HDAg). HDAg bears both nuclear localization signals and RNA-binding motifs and mediates the nuclear import and replication of the HDV RNA (Chou et al., 1998; Dingle et al., 1998). Our results suggest that a cellular counterpart of the HDAg protein may exist in plants. If so, it will be interesting to determine how this protein interacts with viroid RNAs and modulates their biological activities.

We are grateful to Dr David Baulcombe (Sainsbury Centre, UK) for providing the PVX-based vector pP2C2S. We also thank J. Hammond, J. Culver and J. White for critical review of the manuscript.

**References**


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Received 2 January 2001; Accepted 16 February 2001
Published ahead of print (28 February 2001) in JGV Direct as DOI 10.1099/vir.0.17617-0