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Host Involvement in the Replication of Potato Spindle Tuber Viroid and the Evolutionary Relationship Between Plant Viroids and the Hepatitis *Delta* Virus

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(supervisor: Dr. Martin Pelchat)

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
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Abstract

The present study examines the interaction between host RNA polymerase II (RNAP II) and potato spindle tuber viroid (PSTVd), with the goal of locating and characterizing a putative RNAP II promoter within the viroid's RNA genome. By using a co-immunoprecipitation approach coupled with deletion and mutational analysis, RNAP II was shown to specifically bind the left terminal hairpin loop of PSTVd(+) RNA. The interaction with RNAP II appears to be dependent on PSTVd secondary structure features, rather than a particular sequence. These findings provide direct evidence of association between RNAP II and PSTVd RNA, and render a unique example of a possible RNA promoter for RNAP II.

The second part of the study examines the evolutionary relationship between viroids and the hepatitis *delta* virus (HDV), as these pathogens share key structural and functional characteristics. We conclude, based on infection experiments, that HDV and viroids share common strategies and host factors to fulfill their respective life-cycles. We found that both HDV and an HDV mutant lacking the HDAg protein-coding region (miniHDV) can replicate in a plant host. However, miniHDV and PSTVd can replicate in human cells only in the presence of the small *delta* antigen (HDAg-S). Together, these results provide support for the hypothesis that HDV evolved from a viroid-like element through the capture of a cellular transcript necessary for its adaptation to a human host.

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List of Abbreviations

ASBVd – avocado sunblotch viroid

CCCVd – coconut cadang cadang viroid

CChMVd – chrysanthemum chlorotic mottle

CCR – central conserved region

CEVd – citrus exocortis viroid

CTD – carboxy-terminal domain

ELVd – eggplant latent viroid

HBV – hepatitis B virus

HBsAg – hepatitis B surface antigen

HDAg – hepatitis *delta* antigen (refers to both/either form of the protein)

HDAg-L – large *delta* antigen

HDAg-S – small *delta* antigen

HDV – hepatitis *delta* virus

HSVd – hop stunt viroid

INR – initiator sequence

NE – nuclear extract

NEP – nuclear encoded polymerase

PEP – plastid encoded polymerase

PLMVd – peach latent mosaic viroid

PSTVd – potato spindle tuber viroid

RNAP – RNA polymerase

TCH – terminal conserved hairpin

TCR – terminal conserved region

Virp1 – viroid RNA-binding protein 1

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Introduction

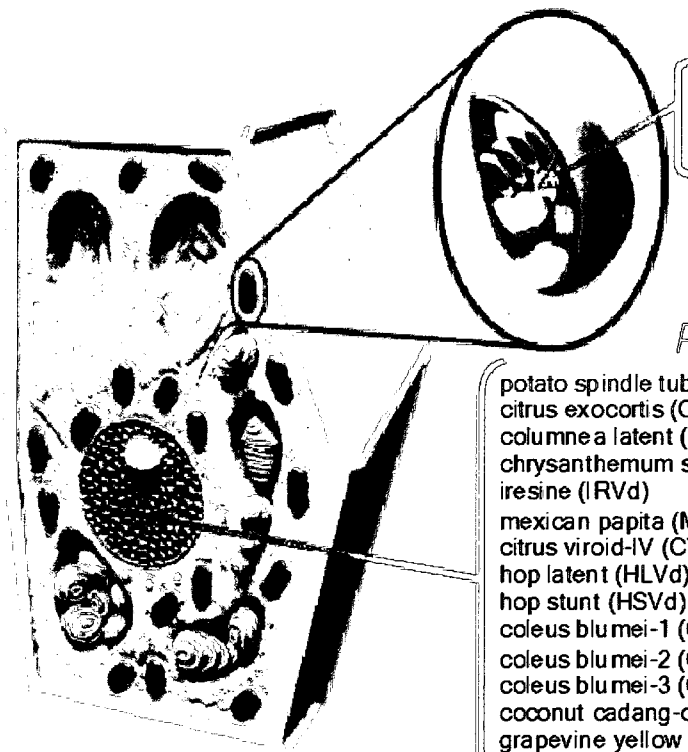
1.1. Viroids

1.1.1. Overview

Up until the early 1970s, it was believed that viruses were the smallest entities capable of causing infectious disease. This notion was changed in 1971 with the discovery of a novel pathogen composed solely of a naked, single-stranded, circular RNA molecule of very low molecular weight (~130kDa). The term viroid was coined for this infectious agent approximately 80 times smaller than the smallest virus known (Diener, 1971). The discovery came about as Diener, a plant pathologist, attempted to identify the cause of a potato disease. Since the discovery of potato spindle tuber viroid (PSTVd), approximately 30 additional viroids having similar characteristics have been discovered. All viroids are subviral RNA pathogens that have the capability to infect higher plants and induce disease, resulting in significant losses in the agriculture industry. As mentioned above, their genomes consist of a single-stranded, covalently closed RNA molecule that, in many cases, adopts an unbranched, rod-like secondary structure due to a high degree of self-complementarity (Sänger *et al.*, 1976). The size of the viroid genome typically ranges between 246 and 401 nucleotides (Flores *et al.*, 2005). Apart from size, viroids are distinguished from RNA viruses by the absence of a protein coat and the absence of protein

coding capacity. This means that viroids must rely on host factors to carry out various tasks associated with their life-cycle. They accomplish autonomous replication and systemic movement within their plant hosts by recruitment of various host proteins via the functional motifs of their RNA genomes. The non-coding viroid RNA, therefore, supplies genetic information such as replicability, pathogenicity, mobility and host-specificity (Tabler and Tsagris, 2004). In addition, some viroids are catalytic RNAs that possess hammerhead ribozyme motifs, which mediate self-cleavage of replicative intermediates during viroid replication. This provides evidence that viroids have evolved independently of viruses, and that they may have an ancient evolutionary origin dating back to the RNA world, thought to have preceded present DNA-based life.

The 31 viroids known to date can be classified into two families, the *Pospiviroidae* family (whose type member is potato spindle tuber viroid (PSTVd)), and the *Avsunviroidae* family (whose type member is avocado sunblotch viroid (ASBVd)). These two families are further subdivided into several phylogenetically related genera (Elena *et al.*, 2001). Most viroids belong to the *Pospiviroidae* family, while the *Avsunviroidae* family consists of only four members (avocado sunblotch viroid (ASBVd), peach latent mosaic viroid (PLMVd), chrysanthemum chlorotic mottle viroid (CChMVd) and eggplant latent viroid (ELVd). This classification is based primarily on the subcellular localization of the viroid within its host plant (see Fig. 1). *Avsunviroidae* family members replicate and accumulate in the chloroplasts, whereas members of the *Pospiviroidae* family replicate in the nucleus, and accumulate in the nucleolus of the host's cells (Flores *et al.*, 2005). Besides viroid localization, several other key structural and functional features distinguish the two families. The RNA genomes of *Pospiviroidae* members adopt an unbranched, rod-like structure, which can be divided into five



Avsunviroidae

avocado sunblotch (ASBVd)
peach latent mosaic (PLMVd)
chrysanthemum chlorotic mottle (CChMVd)
eggplant latent (ELVd)

Pospiviroidae

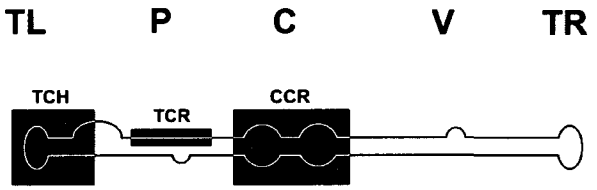
potato spindle tuber (PSTVd)
citrus exocortis (CEVd)
columnea latent (CLVd)
chrysanthemum stunt (CSVd)
iresine (IRVd)
mexican papita (MPVd)
citrus viroid-IV (CVd-IV)
hop latent (HLVd)
hop stunt (HSVd)
coleus blumei-1 (CBVd-1)
coleus blumei-2 (CBVd-2)
coleus blumei-3 (CBVd-3)
coconut cadang-cadang (CCCVd)
grapevine yellow speckle-1 (GYSVd-1)
grapevine yellow speckle-2 (GYSVd-2)
coconut tinangaja (CTiVd)
australian grapevine (AGVd)
apple dimple fruit (ADFVd)
apple scar skin (ASSVd)
citrus bent leaf (CBLVd)
citrus viroid-III (CVd-III)
citrus viroid-OS (CVd-OS)
grapevine 1B (G1BVd)
pear blister canker (PBCVd)
tomato apical stunt (TASVd)
tomato planta macho (TPMVd)
Tomato chlorotic dwarf (TCDVd)

Fig. 1. Viroid localization. Viroid species identified to date, and their subcellular localizations are shown. Members of the family *Avsunviroidae* replicate and accumulate in the chloroplasts, while those belonging to *Pospiviroidae* replicate in the nucleus and accumulate in the nucleolus. (Figure designed by Dr. Martin Pelchat)

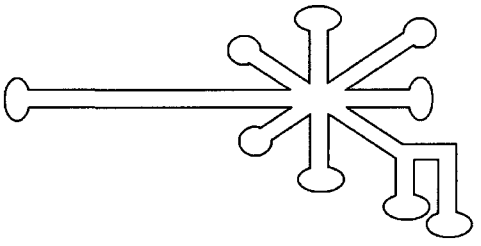
characteristic domains, namely the terminal left (TL), pathogenic (P), central (C), variable (V), and terminal right (TR) domains (Keese and Symons, 1985). Within these domains, there exist several regions that are highly conserved among the different family members. The central conserved region (CCR), located in the C domain, has been shown to be involved in the cleavage and ligation of RNA intermediates during the replication cycle (Baumstark *et al.*, 1997). Pospiviroids also possess either a terminal conserved hairpin (TCH) or a terminal conserved region (TCR), which are located within the TL domain. Although some of the regions (such as CCR and the P domain) have been assigned functions, the function of other domains, including TCH and TCR still remains unclear. Furthermore, the matter is most likely not as simple as initially thought, as some roles seem to be regulated by more than one region (Sano *et al.*, 1992).

Unlike *Pospiviroidae*, members of the *Avsunviroidae* family usually adopt complex branched secondary structures, with many stem-loop regions and do not have distinct conserved domains. However, the definite structure of viroids *in vivo* remains undetermined and it is believed that viroids are capable of adopting alternative RNA conformations during their life-cycle, so as to enable them to interact with host factors and perform the various tasks associated with their replication. *Avsunviroidae* members also all possess hammerhead structures, the simplest known natural ribozymes, and are therefore considered to be catalytic RNAs. A ribozyme is an RNA molecule which displays enzymatic activity, such as endonucleolytic activity in this case. The hammerhead structures of avsunviroids are located on both polarities of the viroid RNA, and mediate *cis* self-cleavage of multimeric RNA intermediates, which are generated during viroid replication (Hutchins *et al.*, 1896; Prody *et al.*, 1986; Foster and Symons, 1987). A schematic representation of key *Avsunviroidae* and *Pospiviroidae* structural characteristics is represented in Fig. 2.

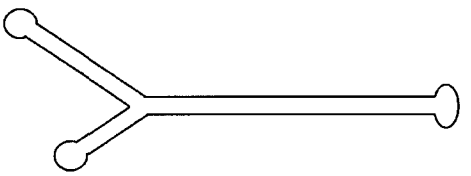
Pospiviroidae



Avsunviroidae



Peach latent mosaic viroid (PLMVd)



Avocado sunblotch viroid (ASBVd)

Fig. 2. Schematic representation of the predicted secondary structures and key features of *Pospiviroidae* and *Avsunviroidae*. Note the five characteristic domains present in *Pospiviroidae* family members: terminal left (TL), pathogenic (P), central (C), variable (V), and terminal right (TR). Also shown are the terminal conserved hairpin (TCH), terminal conserved region (TCR) and central conserved region (CCR).

Finally, the two families differ somewhat in their mode of replication. Although all viroids replicate in their host plants through a rolling-circle mechanism, which involves only RNA intermediates, the *Avsunviroidae* follow the symmetric rolling-circle pathway, whereas *Pospiviroidae* employ the asymmetric pathway (Flores *et al.*, 2004). The replication mechanism of viroids will be discussed in greater detail in the following section.

1.1.2. Viroid replication

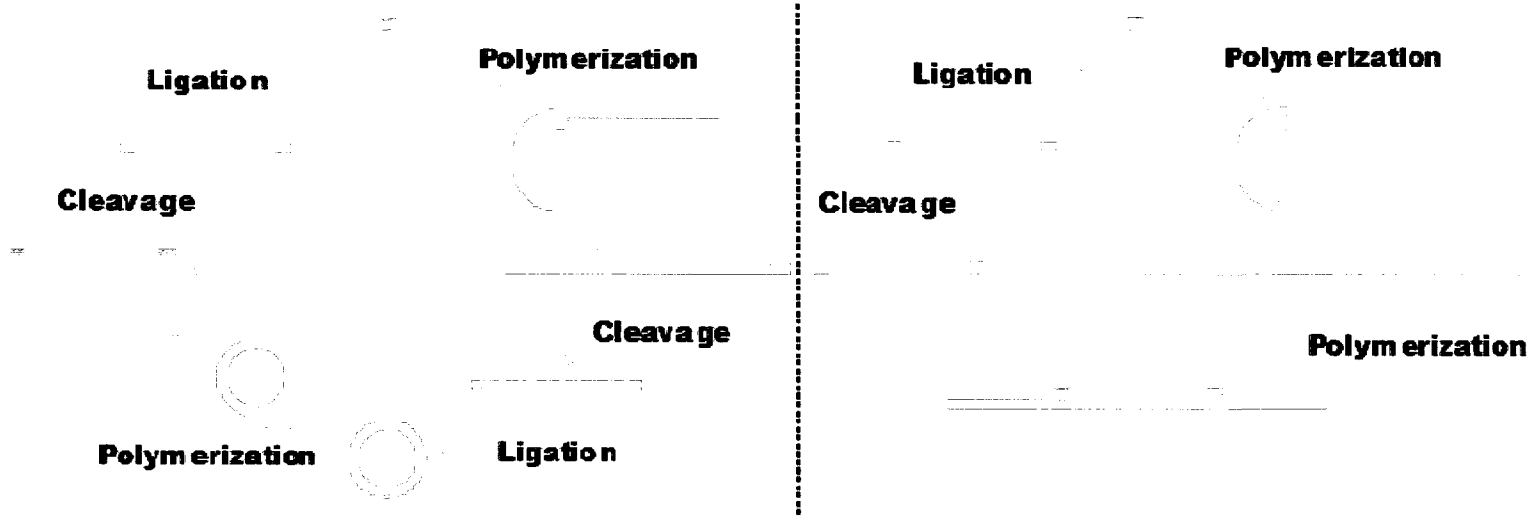
All viroids replicate their RNA genomes via a rolling-circle replication mechanism. Two similar but distinct modes of this replication mechanism exist: the symmetrical pathway (followed by *Avsunviroidae*) and the asymmetrical pathway (followed by *Pospiviroidae*). During rolling-circle replication only RNA species are generated, without any DNA intermediates. By convention, the more abundant polarity of RNA is designated as the (+) strand, while the less abundant, complementary RNA species is designated as the (–) strand. Both the symmetric and the asymmetric rolling-circle pathways begin when the infecting circular monomeric (+) polarity RNA is transcribed, by a host polymerase, into linear multimeric heat-to-tail RNA of (–) polarity. At this point, in the symmetric pathway, the (–) multimers are cleaved into (–) monomers, which are then ligated to produce monomeric (–) circles. The (–) circles serve as templates for the transcription of linear (+) strand multimers, which are then processed (cleaved and ligated) into monomeric (+) circles, thereby completing the replication cycle. In contrast, during asymmetric rolling-circle replication, the linear (–) multimers are not processed. Instead, they directly serve as templates for the synthesis of linear (+) multimers, which are then cleaved

and ligated into monomeric (+) circles. The two pathways of the rolling-circle replication mechanism are schematically depicted in Fig. 3. Even though these major steps of rolling-circle replication are well established and all the replication intermediates have been shown to exist (Bussière *et al.*, 1999), fine detail concerning the viroid-host interactions necessary for the process still lacks.

1.1.3. Host involvement in viroid replication

Since viroids do not encode any proteins, they must rely on host factors in order to carry out processes such as replication, movement and pathogenesis. Much of the past and current research on viroids focuses on these RNA-protein interactions, and the elucidation of the processes that they mediate. Three enzymatic activities are required during the rolling-circle replication mechanism employed by viroids: 1) an RNA-dependent RNA polymerase for generating the multimeric strands, 2) ribonuclease activity for processing them to unit-length linear strands, and 3) an RNA ligase to produce the circular monomers. Members of the *Pospiviroidae* family localize and replicate in the host's nucleus, while those of the *Avsunviroidae* family are confined to the chloroplast. As a result, the members of the two groups must interact with different host factors to perform these activities.

The first step of the rolling-circle replication mechanism requires a host polymerase. Although plants possess RNA-dependent RNA polymerases, they are expressed in the cytoplasm. This is inconsistent with the replication site of viroids, which occurs in the nucleus (*Pospiviroidae*), or the chloroplasts (*Avsunviroidae*) (Flores *et al.*, 2004). This means that viroids



Symmetrical
(*Avsunviroidae*)

Asymmetrical
(*Pospiviroidae*)

Fig. 3. Rolling circle replication mechanism. The (+) polarity strands are depicted in red, the (–) strands are depicted in blue. (Figure designed by Dr. Martin Pelchat)

must be able to convert the template specificity of host DNA-dependent RNA polymerases in order to replicate their own RNA genomes. Previous studies have proposed that the enzyme responsible for the polymerization step in avsunviroids may be either the plastid-encoded polymerase (PEP), or a nuclear-encoded polymerase (NEP). It has been reported that the *E. coli* DNA-dependent RNA polymerase, which is related to PEP, is capable of initiating *in vitro* transcription from a small hairpin structure of PLMVd (termed P11.60) (Pelchat *et al.*, 2001, Pelchat, *et al.*, 2002). This correlation suggests the possibility that PLMVd RNA may be replicated by the bacterial-like RNA polymerase from the peach chloroplast. However, another study demonstrated that the replication of ASBVd is resistant to the bacterial inhibitor tagetitoxin, hinting at the involvement of a NEP, which is structurally very similar to the T7 bacteriophage polymerase (Navarro *et al.*, 2000). Further studies are necessary to clarify the process and identify the exact factors involved.

The enzyme most likely responsible for the polymerization step in pospiviroids is the host DNA-dependent RNA polymerase II (RNAP II). It has been observed that: 1) synthesis of PSTVd can be inhibited by the fungal toxin α-amanitin (Schindler and Mühlbach, 1992), which in low concentrations is a specific inhibitor of RNAP II, but not RNAPs I and III, and 2) citrus exocortis viroid (CEVd), another member of the *Pospiviroidae* family, was found to associate with the largest subunit of RNAP II *in vivo* (Warrilow and Symons, 1999).

The cleavage steps during the replication of pospiviroids are believed to be mediated by host RNases. However, the exact enzymes involved are still under investigation. It has been shown that multimeric PSTVd(+) RNA can be processed to monomeric PSTVd(+) circles in a nuclear extract prepared from healthy potato cells (Tsagris *et al.*, 1987). The enzymes responsible for the cleavage seem to recognize particular secondary structures of the viroid RNA,

rather than a specific sequence, as suggested by the observation that a non-specific fungal RNase is capable of catalyzing the same *in vitro* cleavage and ligation as seen in the nuclear extracts (Tabler *et al.*, 1992). Although RNases can catalyze the cleavage and ligation of pospiviroids *in vitro*, the latter reaction may be mediated by host RNA ligases instead, as suggested by the 2' phosphomonoester, 3',5' phosphodiester bond found at the ligation site, a characteristic of plant RNA ligases (Kiberstis *et al.*, 1985). Members of the *Avsunviroidae* family are capable of self-cleaving their multimeric intermediates into monomeric RNA *in vitro*, without addition of any proteins. The cleavage step is carried out by the endogenous hammerhead ribozyme, which is found on the (+) and (–) strands of the viroid RNA. The ligation step seems to be catalyzed by host ligases, as in pospiviroids. Chloroplast genomes have not been shown to encode any ligases, hence, a nuclear-encoded ligase that localizes to the chloroplast may present an alternate candidate for the ligation reaction. However, self-ligation has also been reported to occur in PLMVd (Côté *et al.*, 2001), evidence stemming from the 2'-5' phosphodiester bonds found at the site of ligation.

1.1.4. Transmission and pathogenesis

It is generally believed that the mechanism of viroid transmission is similar to that of other plant pathogens. The infectious agent may be transmitted from an infected plant, farming tool or insect carrier to a healthy plant via mechanical breaks in the plant tissue. Following initial transmission, the viroid must move to either the nucleus or the chloroplast and begin replication. Accumulation

of viral RNA must be adequately fast in order to escape its host's defense mechanisms. Systemic movement throughout its host ensues. This process most likely occurs via the plasmodesmata, narrow passages that connect neighbouring cells in plant tissues (Ding *et al.*, 1997). Long-distance movement probably proceeds via the phloem, the vascular tissue that transports nutrients throughout the plant (Owens *et al.*, 2001). Most viruses encode particular proteins that facilitate this movement. However, viroids do not encode any proteins and must, therefore, accomplish this feat in a different manner. It is thought that circularity and the intricate secondary structures of viroid RNA provide protection from degradation, as well as a platform for the recruitment of host proteins that may facilitate the movement process (reviewed by Tabler and Tsagris, 2004). Two studies have demonstrated that hop stunt viroid (HSVd) interacts and forms a ribonucleoprotein complex with phloem protein 2 (Owens *et al.*, 2001; Gomez and Pallas, 2001). Another study has demonstrated that the long-distance movement is not just due to bulk flow through the phloem, but rather a coordinated process involving the interaction of host protein(s) with specific viroid RNA motifs (Zhu *et al.*, 2002). In this study, a mutated PSTVd was able to enter the phloem of *N. tabacum* and spread through it, but not exit.

Numerous studies have focused on the identification and characterization of viroid-protein interactions and their significance in viroid propagation. One protein of interest is the viroid RNA-binding protein 1 (Virp1), which was demonstrated to interact with the (+) polarity of PSTVd (Sägesser *et al.*, 1997). This protein interacts specifically with several PSTVd-related viroids, but not with other RNAs. Virp1 is a transcriptional regulator and contains a nuclear localization signal. It is therefore tempting to suggest that Virp1 plays a role in recruiting viroid RNA to the nucleus and mediating its interaction with the host's transcriptional machinery. Using a PSTVd mutant displaying impaired interaction with Virp 1, the binding site for the

protein was mapped onto the right terminal domain of the viroid and designated as the RY motif (Maniataki *et al.*, 2003). Disruption of the RY motif was confirmed to be detrimental to infectivity, suggesting the vital role of Virp1 in the viroid life-cycle (Gozmanova *et al.*, 2003).

To date, the exact molecular mechanisms by which viroids cause disease remain unclear. Once again, pathogenicity appears to be the result of intricate interactions between viroid RNA motifs and host factors. In an attempt to identify exactly which regions of the viroid genome dictate the severity of the disease symptoms, the pathogenicity domain was shown to play a critical role. The severity of a viroid infection could be attributed to the thermodynamic stability and the conformation of the pathogenic domain (Owens *et al.*, 1996). The stability of this region was also shown to be inversely related to severity of disease (Schnölzer *et al.*, 1985). However, pathogenicity seems to be a more complex matter than initially believed. One study using chimeras constructed by exchanging domains between two *Pospiviroidae* members has determined that complex interactions of three discrete regions within multiple domains regulate viroid pathogenicity (Sano *et al.*, 1992).

Although the exact mechanisms by which viroids induce disease remain a mystery, involvement of a number of host proteins and their potential roles in the process are beginning to emerge. One such protein is P68, a protein kinase, which was shown to be phosphorylated and activated by PSTVd. Furthermore, the degree of activation was directly correlated to the severity of the viroid strain, suggesting a role for this interaction in triggering pathogenicity (Diener *et al.*, 1993). Viroids of the *Pospiviroidae* family have also been shown to accumulate in the nucleoli of their hosts' cells, where they may have an effect on small nucleolar RNAs and rRNA processing by competing for nucleolar proteins (Qi and Ding, 2003). In addition, targets of viroids may not exclusively be host proteins, but nucleic acids as well. Viroids have been shown

to contain regions complementary to certain cellular RNAs. For instance, a region of apple scar skin viroid (ASSVd) and PSTVd contains a sequence similar to that of U1 RNA (Hashimoto and Koganezawa, 1987; Diener, 1981), while other viroids have regions partially complementary to the 7S RNA, a plant equivalent of the human 7SL RNA, which is an integral component of the signal recognition particle (SRP) that mediates protein translocation (Haas *et al.*, 1988). This would suggest that viroids may exert their pathogenic effects via a *trans*-acting ribozyme effect, or by RNA silencing, thereby inhibiting the expression of some host genes. It has been shown that both families of viroids are capable of generating small RNAs of the size expected for siRNAs in their hosts (Itaya *et al.*, 2001; for a review on viroids and RNA silencing see Tabler and Tsagris, 2004). Clearly, there are still many unanswered questions regarding viroid pathogenesis and likely more than just one mechanism exist.

1.1.5. Symptoms of viroid-caused diseases

Viroids are the causative agents of serious diseases in a number of economically important crops such as potato, tomato, hop, grapevine, coconut, citrus, *etc.* Some viroids of the *Pospiviroidae* family have a variety of host plants, while others, typically members of the *Avsunviroidae* family, may just have one natural host. Often, very subtle changes to a viroid's genome, such as a single nucleotide substitution, may have an astounding effect on its virulence, or alter its host specificity (Wassenegger *et al.*, 1996). The symptoms of the same viroid infection may drastically differ in different hosts, and may range from mild or asymptomatic to severe

depending on the variant (reviewed by Flores *et al.*, 2005). Perhaps the most drastic example of disease caused by a viroid is the effect of coconut cadang cadang viroid (CCCVd) infection, which is responsible for the death of millions of coconut trees in the Philippines each year. Other common symptoms of viroid infections are similar to those of plant virus infections and include: discoloration, spotting, necrosis or dwarfing (leaves), scaling, cracking and cankers (bark), cracking and deformations (fruit), as well as more general effects such as delay and changes in the regular growth pattern (Flores *et al.*, 2005).

1.1.6. Potato spindle tuber viroid (PSTVd)

Potato spindle tuber viroid (PSTVd) became the first viroid discovered, when it was identified as the causative agent of potato spindle tuber disease (Diener, 1971). Since its initial discovery, PSTVd has remained an important model and subject of a significant amount of research aimed at elucidating the nature of these unique RNA pathogens. It is the type-member of the *Pospiviroidae* family. The PSTVd genome consists of a 359nt-long single-stranded, circular RNA molecule. The PSTVd genome depicting the predicted secondary structure and the five characteristic domains found in pospiviroids is schematically represented in Fig. 4.

Natural hosts of PSTVd include the potato (*Solanum* sp.) and the tomato (*Lycopersicon* sp.), although infections in a wide variety of other plants have been experimentally induced in the past. (Hailstones *et al.*, 2003) The severity of PSTVd infection can vary greatly from one strain of the viroid to the next and ranges from mild or asymptomatic to severe. It has been found

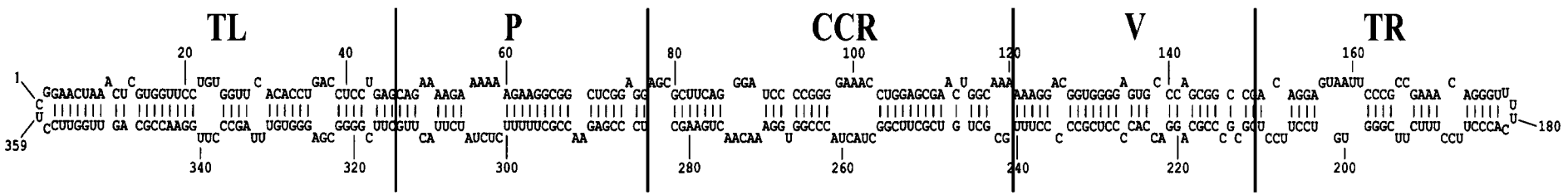

TL
P
CCR
V
TR
1
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
359

Fig. 4. Schematic representation of the potato spindle tuber viroid (PSTVd) RNA genome. Sequence and secondary structure are shown. Note the five characteristic domains present in *Pospiviroidae* family members, terminal left (TL), pathogenic (P), central conserved region (CCR), variable (V), and terminal right (TR).

that certain environmental conditions such as hot climates can further exacerbate the severity of the infection. Some of the well documented symptoms of PSTVd infection in potato plants include: upright, stunted and thinner plants, smaller and distorted leaves, as well as elongated, narrow, spindle-like tubers that may develop knobs, swelling, cracking, and numerous prominent "eyes". It has been reported that PSTVd may be responsible for up to 64% of yield losses of potato crops (information from: http://www.aphidwatch.com/potato/potato-spindle-virus.pdf)

1.2. Hepatitis *delta* virus (HDV)

1.2.1. Overview

The hepatitis *delta* virus (HDV) was first identified in 1977 with the discovery of a novel nuclear antigen present in the hepatocytes of patients chronically infected with hepatitis B (HBV). This antigen was termed the "*delta*" antigen. It was only found in association with HBV infection, and closely resembles the hepatitis B core antigen (HBcAg) in its subcellular localization (Rizzetto *et al.*, 1977; Bonino *et al.*, 1984). This antigen was later shown to be a component of a novel RNA virus that requires co-infection with HBV for transmission by using the hepatitis B surface antigen (HBsAg) for its own virion coat (Taylor, 1996; Lai, 1994). HDV is, therefore, considered a defective satellite virus of HBV. It is the smallest RNA pathogen known to infect humans and the disease it causes is termed hepatitis D. Although humans are a natural host for the virus, it has been experimentally transmitted to chimpanzees infected with HBV and woodchucks infected with the woodchuck hepatitis virus (Negro, 1996). The virus has been found responsible for some of the most severe forms of hepatitis in HBV-infected patients. HDV co-infection aggravates the symptoms and disease progression of HBV and has been associated with the development of fulminant hepatitis, hepatocellular carcinoma, cirrhosis of the liver and liver failure (Govindarajan et al., 1984; Jacobson et al., 1985; Kanel et al., 1984; Romeo et al., 2009). A recent study reports that 80% of patients who are chronically infected with HDV will experience cirrhosis of the liver within five to ten years (Koytak et al., 2007).

HDV is spread via infected blood and blood products through bloodborne, sexual, percutaneous, and more rarely through perinatal contact (World Health Organization (WHO), 2001). Since the presence of HBV is a requirement for its propagation, inoculation with the HDV viral genome alone will not result in hepatitis D infection in individuals not infected with HBV. Although the HDV RNA will be able to replicate, virion particles will not be released. However, chronic HBV carriers and persons not immunized against HBV have an increased risk of HBV infection with either immediate or later infection with HDV (WHO, 2001).

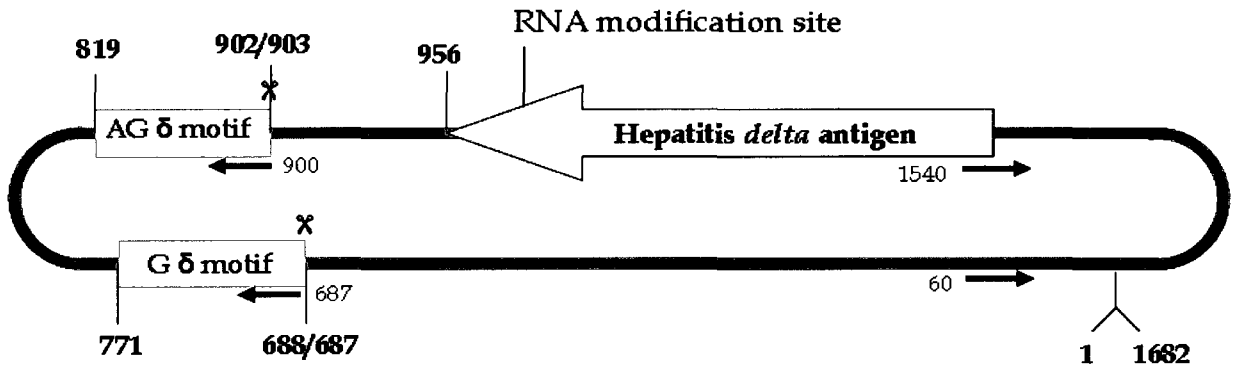
HDV is present throughout the world with a distribution that closely resembles that of HBV infection. Its prevalence is highest in Russia, Romania, some Mediterranean countries and in tropical regions (Lai, 1994). About two billion people have been exposed to HBV and 350 million people suffer from chronic HBV infection (data acquired in 2006; www.cdc.gov). Around 10% of HBV-infected individuals are also co-infected with HDV (www.cdc.gov).

Currently, there is no cure or effective antiviral treatment for the disease. Liver transplantation may be considered as an option for fulminate acute and end-stage chronic HDV infection. Administration of large doses of α-interferon has also shown slight improvement in disease symptoms of HDV infection (Lau *et* al., 1999). Since HDV is dependent on HBV, prevention and control of HBV remains key in preventing the spread of HDV. Although vaccination against HBV is imperative in preventing HBV-HDV co-infection, it does not offer protection against HDV to chronic carriers of HBV (WHO, 2001).

1.2.2. Molecular biology of HDV: the HDV genome and HDAg

HDV is composed of a single-stranded circular RNA molecule that is 1682nt long (Chen et al., 1986). The genome can replicate autonomously within the host cells, but the presence of HBV as a helper virus is crucial as HBV surface antigens are necessary for virus particle production (see Fig. 5). HDV RNA exists in two forms inside its host's cells, the genomic and the antigenomic form. The genomic form of HDV is approximately 10 times more abundant than the antigenomic form (Chen *et al.*, 1986). The two polarities of HDV are most abundant as monomers, but dimmers and other oligomers also exist at detectable levels. In fact, this was one of the first clues suggesting that HDV replicates by a rolling-circle mechanism (Branch and Robertson, 1984). The HDV genome, which has been cloned and sequenced in 1986, is not related to the genome of HBV or to that of any other hepadnavirus. Hence, it is classified on its own as a Deltavirus (Wang *et al.*, 1986; Lai, 1995). In fact, this very unique human pathogen shares many similarities with viriods and viroid-like plant RNA satellites. Common characteristics exist between HDV and viroids in terms of their genome structure and function, and their replication mechanism. Similarly to the *Pospiviroidae* family of viroids, the genome of HDV adopts an unbranched rod-like secondary structure under native conditions due to a high degree of canonical intramolecular base pairing (Wang *et al.*, 1986, see Fig. 5). In fact, examination of the HDV genome sequence reveals that it is partially related to that of viroids, suggesting a common evolutionary origin (Elena *et al.*, 1991). Viroids and HDV also share a common replication strategy, the rolling-circle replication mechanism (further discussed in the next section). Similarly to the *Avsunviroidae* family of viroids, HDV possesses a ribozyme motif on both polarities of its

A


RNA modification site
819
902/903
956
AG δ motif
900
Hepatitis *delta* antigen
1540
G δ motif
687
60
771
688/687
1
1682

B

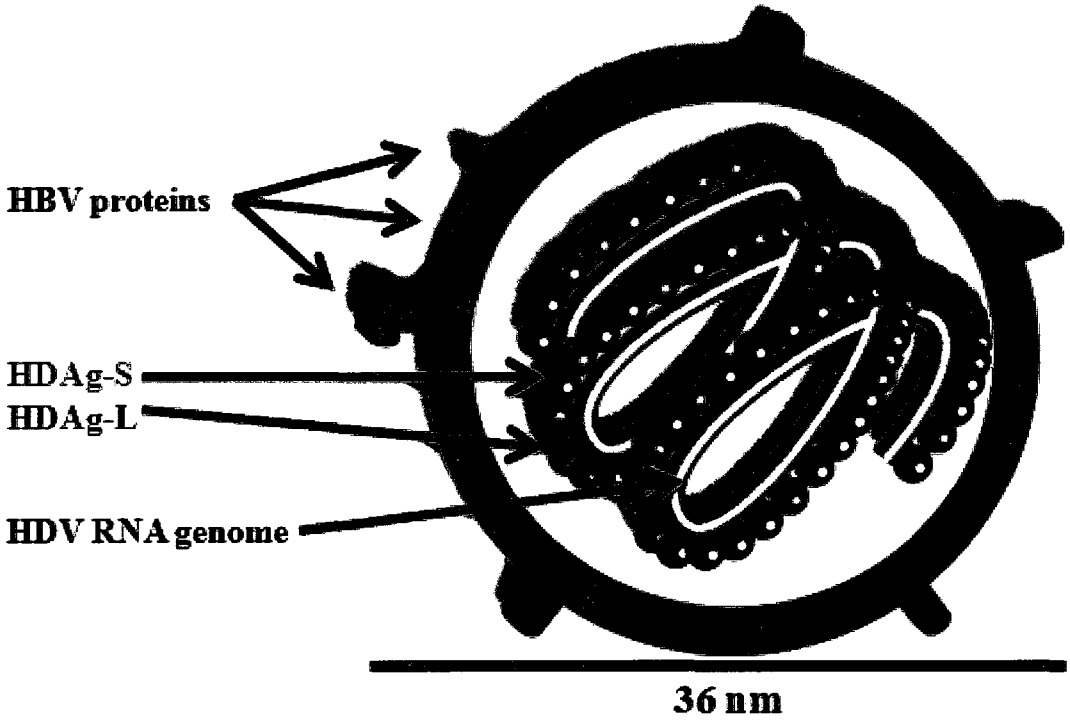

HBV proteins
HDAg-S
HDAg-L
HDV RNA genome
36 nm

Fig. 5. The hepatitis *delta* virus (HDV). (A) HDV RNA genome is represented in its circular, rod-like structure. The two ribozyme motifs and the opening reading frame of the *delta* antigens are shown. (B) Schematic representation of a hepatitis *delta* virion particle. (Figure designed by Dr. Martin Pelchat)

genome. These *delta* ribozymes are responsible for the *cis* cleavage of HDV RNA multimeric intermediates during the virus' replication cycle (Wu *et al.*, 1989, see Fig. 6).

Despite these parallels with viroids, HDV possesses some unique characteristics. Most notably, the genome of HDV is several times longer than that of an average viroid (~1.7 kilobases for HDV versus ~250-400 bases for viroids). This is due to a protein-coding sequence present on the HDV genome. HDV possesses one open reading frame (ORF), which encodes two proteins: the large and small *delta* antigens, termed HDAg-L and HDAg-S, respectively. HDAg-L (214aa long, 27kDa) arises due to a post-transcriptional RNA-editing event which modifies the HDAg-S (195aa long, 24kDa) UAG stop codon to a UGG (tryptophan), resulting in the production of a 19-amino acid longer protein (Casey and Gerin, 1995; Lai, 1995). Although the two protein forms share common functional motifs (see Fig. 7), they have very distinct roles in the virus' life-cycle. HDAg-S is produced early in infection and is necessary for RNA replication, whereas HDAg-L is produced in the later stages of infection and is important for virion assembly (Kuo *et al.*, 1989; Chang *et al.* 1991). HDAg-L also has a role as a negative regulator of HDV RNA replication (Chao *et al.*, 1990). The *delta* antigens undergo various post-translational modifications thought to be imperative for their function in the HDV life-cycle. These modifications are depicted in Fig. 7, and include phosphorylation, methylation, acetylation and isoprenylation (reviewed by Lai, 2005). Isoprenylation of HDAg-L has been shown to be essential in virion assembly (Glen *et al.*, 1992).

The HDV virus particle envelope contains lipids and the small, medium and large forms of the hepatitis B surface antigen, which is necessary for the encapsidation of the HDV genome. Inside this coat, HDV is found in the form of a ribonucleoprotein (RNP) complex that consists of

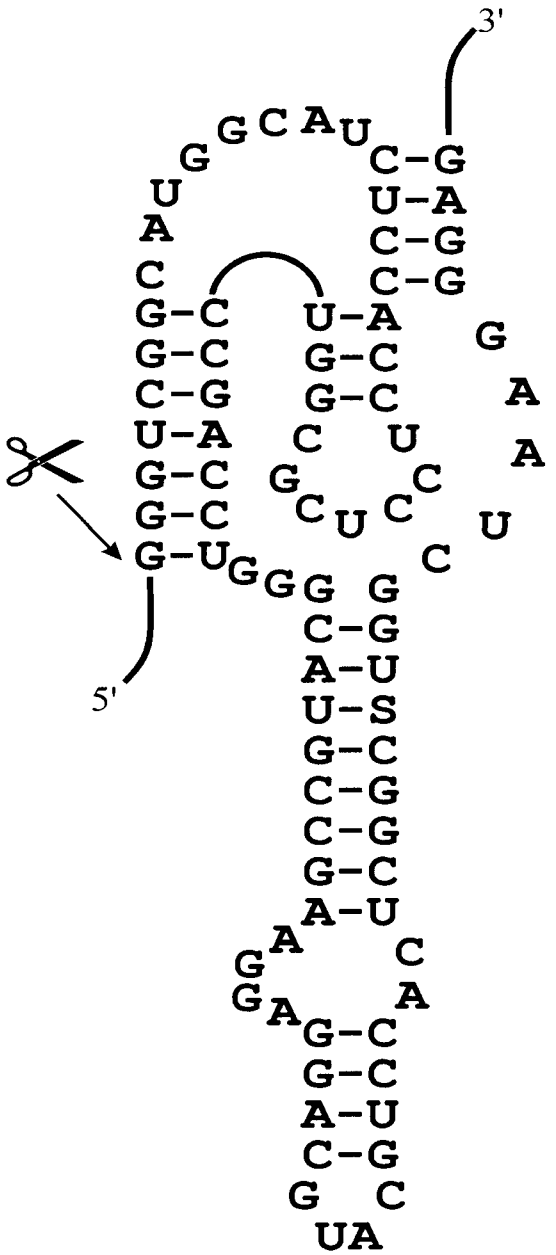
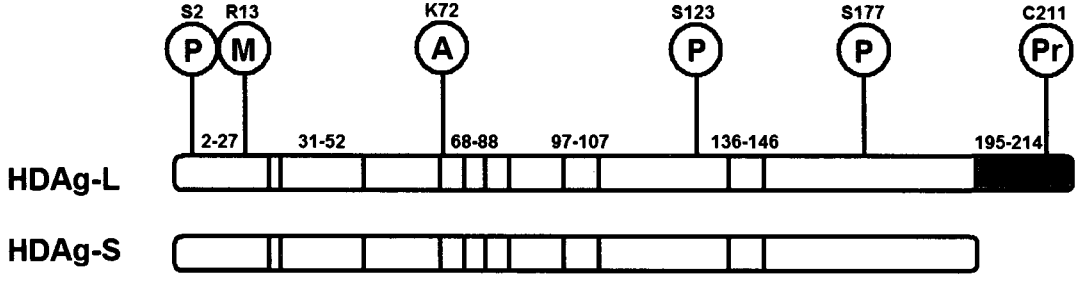

3'
5'

Fig. 6. Schematic representation of the *delta* ribozyme. Secondary structure of the *cis*-acting ribozyme present on the antigenomic strand of HDV is shown. (Figure designed by Dr. Martin Pelchat)



- RNA-binding domain (RBD)
- Coiled-coil sequence (CCS)
- Nuclear localization sequence (NLS)
- Helix-loop-helix (HLH)
- Virus assembly signal (VAS)

Fig. 7. The HDV-encoded large (HDAg-L) and small (HDAg-S) *delta* antigens. Functional domains and posttranscriptional modifications are shown (P – phosphorylation; M – methylation; A – acetylation; Pr – prenylation). (Figure adapted from Lai, 2005)

the HDV circular genomic RNA and about 60 copies of HDAg (reviewed by Sureau, 2006; Bonino *et al.*, 1984).

1.2.3. The HDV life-cycle

HDV infection begins with the virion particles entering the bloodstream and migrating to the liver. The HDV particles then enter the hepatocyte. Exactly how this is accomplished is still unknown, but it is likely to be mediated by the interaction between the preS1 domain of the hepatitis B surface antigen (HBsAg, an HBV protein component of the HDV envelope) and a yet unidentified hepatocyte surface receptor (Taylor, 2003). Next, the HDV RNA genome is uncoated and transported to the nucleus. At this point, the RNA is still bound by HDAg proteins, which may aid in preventing degradation. HDAg-S may also be involved in directing the HDV RNA to the nucleus (Chang *et al.* 1988; Lai, 1995). The host nucleus is the site of HDV replication. Like viroids, HDV replicates via a rolling-circle mechanism. More specifically, HDV follows the symmetrical pathway of the rolling-circle replication mechanism, similarly to the *Avsunviroidae* family of viroids. Briefly, the genomic circular RNA is transcribed by a host polymerase into multimeric, linear RNA molecules of the antigenomic polarity. This RNA species is self-cleaved by the intrinsic *delta* ribozyme into unit-length RNA, which is then ligated, most likely by yet unidentified host factors, into circular antigenomic HDV RNA. The process is then repeated to generate the genomic RNA once again. During this process, the transcription of a subgenomic HDV mRNA, which encodes HDAg-S and HDAg-L, from the

genomic strand also occurs. These events are depicted in Fig. 8, and are discussed in greater detail below.

The rolling-circle replication mechanism is a process that involves RNA-templated RNA replication, without any DNA intermediates. Most RNA viruses carry out this process with the aid of a virus-encoded RNA-dependent RNA polymerase (RdRP). However, HDV does not encode its own RdRP, and neither do its hosts. To date, no mammalian RdRP ortholog has been found. Numerous studies have been conducted with the aim of identifying the host polymerase responsible for the replication of HDV RNA. Nearly two decades ago, it was proposed that the enzyme responsible for replicating HDV is the host's DNA-dependent RNA polymerase II (RNAP II). Such studies involved the use of α-amanitin, a toxin obtained from the *Amanita phalloides* mushroom, which is a specific inhibitor of RNAP II in very low doses. It was shown that HDV RNA replication was inhibited by low doses (1-5μg/mL) of the toxin (Macnaughton *et al.*, 1991). Typically RNAP II uses only DNA templates, and is associated with the transcription of mRNA from DNA. However, the role of RNAP II has been revised to include some unique exceptions to the rule, such as some viroids. Since then, various studies have provided support for RNAP II as a candidate for HDV replication. In 1993, Taylor's laboratory demonstrated that HDV can replicate *in vitro* in a hepatocyte nuclear extract, and that this replication could be inhibited upon addition of α-amanitin or an anti-RNAP II antibody (Fu and Taylor, 1993). More recently, it has been shown that replication of HDV became resistant to α-amanitin in a cell line that expresses an α-amanitin-resistant RNAP II (Modahl *et al.*, 2000). Our laboratory has recently provided direct evidence that HDV RNA interacts specifically with host RNAP II, and has assigned the location of the putative RNAP II promoter to the terminal hairpin loops of both senses of the HDV RNA (Greco-Stewart *et al.*, 2007).

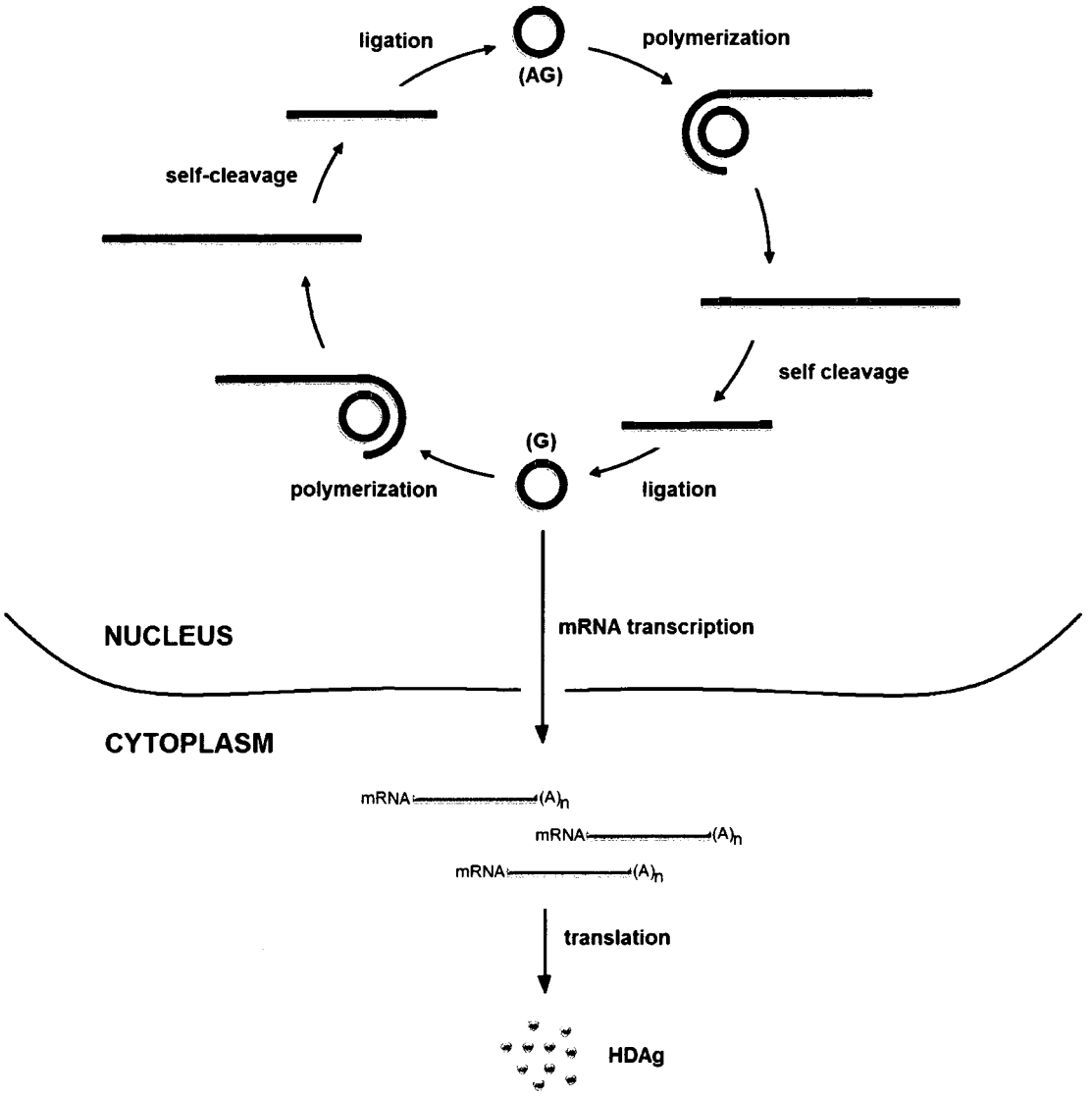

ligation
(AG)
polymerization
self-cleavage
self cleavage
(G)
polymerization
ligation
NUCLEUS
mRNA transcription
CYTOPLASM
mRNA
(A)n
mRNA
(A)n
mRNA
(A)n
translation
HDAg

Fig. 8. A model of the HDV replication cycle. RNAs of genomic polarity are depicted in red, while those of antigenomic polarity are shown in blue.

However, despite these findings, data have emerged that suggest that RNAP II may not be the only polymerase involved in the HDV life cycle. More specifically, the genomic and the antigenomic strands may interact with distinct host polymerases, as they seem to have different sensitivities to α-amanitin. The synthesis of the genomic strand from antigenomic RNA, as well as the transcription of the 0.8kb HDAg mRNA, are quite sensitive to very low doses of the toxin. However, production of the antigenomic strand from genomic RNA is resistant to very high doses of α-amanitin (up to 100μg/mL) (Macnaughton *et al.*, 2002). Overall, these data indicate that HDV replication is somewhat more complex than initially thought and more research is needed to determine exactly how the replication of the genomic and antigenomic strands differ. Perhaps the synthesis of the antigenomic strand occurs in a different subnuclear compartment that is impervious to α-amanitin, or distinct polymerases are used for the different strands. Our laboratory has very recently shown that HDV RNA interacts with the human RNA polymerases I and III (Greco-Stewart *et al.*, 2009).

1.3. Statement of purpose

The first part of this project focuses on PSTVd and attempts to identify and characterize its interactions with host proteins. The main focus of the project is the interaction between PSTVd and the enzyme believed to be responsible for its replication, the host DNA-dependent RNA polymerase II (RNAP II). **We hypothesize that RNAP II catalyzes the polymerization steps during PSTVd replication by recognizing a specific sequence/secondary structure on the PSTVd RNA genome, which it uses as a promoter for RNA-templated RNA transcription.** This hypothesis is based mainly on two previous studies, in which it has been observed that: 1) synthesis of PSTVd is inhibited by low concentrations of the fungal toxin α-amanitin, which is a specific inhibitor of RNAP II, but not RNAPs I and III (Schindler and Mühlbach, 1992), and 2) citrus exocortis viroid (CEVd), another member of the *Pospiviroidae* family, was found to associate with the largest subunit of RNAP II in the tomato (Warrilow and Symons, 1999). **The objectives of the study were: 1) to determine whether RNAP II from tomato nuclear extract (NE) binds PSTVd RNA, 2) to narrow down the region(s) of the PSTVd genome to which RNAP II binds (i.e. to define a promoter for RNAP II), and 3) to characterize the promoter features (i.e. sequences and structural motifs) necessary for the interaction.** The approaches used to accomplish these goals include co-immunoprecipitation experiments with *in vitro*-synthesized PSTVd-derived RNAs and an RNAP II-containing tomato nuclear extract, combined with deletion analyses and a mutational approach.

Overall, this project will help to elucidate the replication mechanism of pospiviroids, and may also give us clues about the replication of other members of the brotherhood of the small

self-replicable RNA pathogens (i.e. Hepatitis *delta* virus, viroid-like plant satellite RNAs, defective interfering RNAs, etc). Furthermore, this unique *in vitro* system is ideally suited for the analysis of RNA amplification by DNA-dependent RNA polymerase. Finally, the study may also help us gain insights into the evolutionary relationship between RNA and DNA.

The second part of the project focuses on HDV and examines its parallels and similarities to plant viroids in an attempt to understand more about the evolutionary origin of this unique human pathogen. The idea that HDV and plant viroids are evolutionarily related has been around for a number of years (Elena *et al.*, 1991). It has been proposed that HDV may have evolved from an ancient viroid-like element via the capture of a cellular transcript, thereby allowing it to adapt to and acquire a new host. The present study aims to provide supporting evidence concerning this notion of relationship between HDV and viroids. **We hypothesize that if HDV and plant viroids are evolutionarily related, they may still share the same mechanisms by which they propagate and have common host factors with which they interact to complete their life-cycles.** To explore this idea we plan to study the effects of these two pathogens on non-conventional hosts. Replication of PSTVd RNA in human cells, as well as replication of HDV RNA in tomato plants, will be examined. In addition, the effects of an HDV mutant lacking the protein-coding region (miniHDV), which bears a striking resemblance to viroids, on both plants and human cells will be investigated. Furthermore, RNA affinity chromatography will be used in an attempt to identify host interactors that are common to PSTVd and HDV. **The main objectives of this study are to assess the host and pathogen requirements necessary for PSTVd and HDV infection, and to identify potential common factors that play a role in both of these pathogens' survival strategies.**

The exact details concerning the origin and propagation strategies of viroids and HDV have been the subject of much research effort since their initial discovery. However, our knowledge is far from complete and many questions remain unanswered. We hope that our research will provide some information towards a better understanding of this small group of RNA pathogens. Deeper comprehension of the mechanisms by which HDV and viroids operate could lead to the development of better treatments for the diseases they cause, and perhaps even more importantly, to a more profound understanding of RNA structure and function in general.

Materials and Methods

2.1. Synthesis of PSTVd-derived RNAs.

E.coli XL-1 Blue $CaCl_2$ competent cells (Invitrogen) were transformed with a pBluescript II KS derivative containing a trimeric PSTVd cDNA insert using the heat shock method, and grown overnight at 37 °C. In brief, 100μL of competent cells were incubated with 0.1μg plasmid DNA on ice for 30 minutes. The cells were heat shocked for 50 seconds at 42 °C and placed on ice for 50 seconds. The cells were then incubated in 1mL LB broth (Fisher) for 2 hours with agitation at 37°C and plated on ampicillin (100μg/mL) plates. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen). Polymerase chain reaction (PCR) using Vent polymerase (New England Biolabs; NEB) and isolated plasmid as template was employed for the amplification of DNA fragments corresponding to selected regions of the PSTVd genome. The T7 promoter sequence was incorporated into the appropriate PSTVd-specific primers used in the PCR reaction in order to allow for subsequent synthesis of PSTVd RNA fragments of either (+) or (–) polarity (refer to Appendix B for primer sequences). PCR products were run on 1% w/v agarose gel in 1X TBE buffer (100mM Tris-borate, pH 8.3, 1mM EDTA). Following SYBR green or ethidium bromide staining and visualization by ultraviolet light, the correct DNA fragments were excised and eluted using the QIAquick Gel Extraction Kit (Qiagen). DNA fragments were then used as templates for *in vitro* run-off transcription with T7 RNA polymerase (NEB). Transcription reactions were prepared by adding: 20μL of 5X transcription buffer (400mM Tris-HCl (pH 7.9), 200mM dithiothreitol, 120mM $MgCl_2$, 10mM spermidine), 5μL of 25mM NTPs, 2μL of T7 polymerase, 1μL of RNA guard (Amersham), 1μL of 100X pyrophosphatase, 20μL of DNA

template, and 51μL of H_2O per microcentrifuge tube. Transcription reactions were incubated at 37°C for 4 hours, followed by a 30 minute treatment with 1μL DNase I (Promega) to remove template DNA. The RNA products were electrophoresed on denaturing 5% polyacrylamide gel containing 3.5M urea in 1X TBE buffer. Following electrophoresis, RNA bands were visualized by UV shadowing, excised, and eluted at room temperature for 3 hours in RNA elution buffer (500mM ammonium acetate, 0.1% SDS, 20mM EDTA), ethanol precipitated, and resuspended in H_2O. RNAs were further cleaned by passage through Sephadex G-50 (Amersham Pharmacia Biotech) columns, followed by ethanol precipitation and resuspension in H_2O. Concentration of RNA was determined by spectrophotometry at 260nm, and quality was verified by running an aliquot on 10% polyacrylamide gel, followed by methylene blue staining.

2.2. Radiolabeling of RNAs.

10pmol of *in vitro*-synthesized RNA was 5'-end radiolabeled with [γ-^{32}P]ATP (Perkin-Elmer). First, the RNA was incubated with 2μL calf intestinal phosphatase (CIP) (NEB), 2μL NE Buffer 3 (10X) (NEB) and 1μL RNA Guard (Amersham) for 30 minutes at room temperature, followed by phenol/chloroform extraction and ethanol precipitation. The RNA was rephosphorylated by incubation with 2μL T4 polynucleotide kinase (T4 PNK) (NEB), 2μL T4 PNK Buffer (NEB) and 1μL RNA Guard, in the presence of [γ-^{32}P]ATP. Incubations were performed at 37°C for 1 hour, and followed by phenol/chloroform extraction, passage through a G-50 sephadex column, ethanol precipitation, and resuspension in 20μL H_2O. Alternatively, *in vitro* T7 transcription was carried out in the presence of [α-^{32}P]GTP (Perkin-Elmer), as previously described.

2.3. Preparation of tomato nuclear extract.

Healthy young tomato leaves were ground to a fine powder in liquid nitrogen, and nuclear extract was prepared using the CelLytic PN Plant Nuclei Isolation/Extraction Kit (Sigma-Aldrich) in accordance with the manufacturer's protocol. PMSF was added to inhibit endogenous proteases. Protein concentration was determined by Bradford assay (BioRad). The presence of DNA-dependent RNA polymerase II (RNAP II) was verified by Western blot analysis (using One-Step™ Western Kit (GenScript Corporation)) with a mouse monoclonal antibody directed against the largest subunit of RNAP II (clone H14; Abcam). Nuclear extract was flash-frozen and stored at -80°C.

2.4. Co-immunoprecipitations.

The Protein-G Immunoprecipitation Kit (Sigma-Aldrich) was used to co-immunoprecipitate PSTVd RNAs as specified by the manufacturer, with minor modifications to the protocol. In brief, 3µL of radiolabeled RNA (approximately 1pmol RNA) was incubated with 30µg of tomato nuclear extract in RIPA buffer (50mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 1mM EDTA, 150mM NaCl) in spin columns for 30 minutes at 4°C. A 50-fold molecular excess of either tRNA or P11.60 RNA was added to some reactions as non-specific competitors, and a 50-fold excess of non-radiolabeled homologous RNA was employed as a specific competitor. 5µg of mouse monoclonal antibody directed against the C-terminal domain of the largest subunit of RNAP II (clone H14; Abcam) was then added to each reaction and incubated for an additional 2 hours at 4°C on a shaker. 50µL of pre-washed protein-G

agarose beads were added to the reactions and incubated for 4 hours to overnight at 4°C. Following incubation, samples were washed 4 times with 700µL of RIPA buffer, and eluted by incubation at 90°C for 10 minutes with acrylamide loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 80% formamide, 10mM EDTA (pH 8.0)) , followed by centrifugation. Eluted samples were electrophoresed on denaturing 5% polyacrylamide gels cantaining 3.5M urea, and RNA was detected by autoradiography or phosphor imaging.

2.5. Isolation of Total RNA

Total RNA was extracted from either fresh or frozen plant leaves using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen), and following the protocol for purifying RNA from plant tissues. Briefly, plant leaves were ground to a fine powder with a mortar and pestle in liquid nitrogen, lysed with Lysis buffer (Invitrogen) and passed through homogenizer columns (Invitrogen). Samples were then ethanol precipitated, washed and eluted as specified in the manufacturer's protocol and flash-frozen in liquid nitrogen and stored at -80°C. Total RNA from HepG2 cells was extracted using the same kit, but following the protocol for purifying RNA from animal cells.

2.6. RT-PCR

First strand cDNA syntheses were performed using SuperScript™ III (Invitrogen), 1µg total RNA sample purified from tomato leaves and 10pmol of appropriate primer, by following the manufacturer's protocol. The obtained first strand cDNA was then amplified directly by PCR

using Platinum® *Taq* DNA Polymerase (Invitrogen). Products were analysed by agarose gel electrophoresis.

2.7. RNA affinity chromatography.

200pmol RNA was oxidized by incubation in 20mM Tris-HCl pH 7.5 and 10mM Na-m-periodate at 4°C for 1 hour in the dark. Oxidized RNA was ethanol precipitated, resuspended in 60µL of 0.1M sodium acetate, pH 5, and incubated with 600µL of pre-washed adipic acid dihydrazide agarose beads (Sigma-Aldrich) overnight at 4°C on a rotator in order to couple the RNA to the beads. Unbound RNA was removed by centrifugation and washing 3 times with binding buffer (10mM Tris-HCl, pH 7.4, 10mM KCl, 150mM NaCl, 1mM $MgCl_2$, 5% glycerol, RNA guard) and 3 times with 1X IP buffer (Sigma-Aldrich). 100µL of potato nuclear extract was then added to each reaction, and incubated for 30 minutes at room temperature on a rotator. Unbound proteins were removed by centrifugation and washing of each reaction 4 times with 700µL of binding buffer. Samples were eluted by heating at 90°C for 10 minutes with SDS loading buffer (100mM Tris-HCl (pH 6.8), 4%w/v SDS, 0.2%w/v bromophenol blue, 20%v/v glycerol), and electrophoresed on 10% SDS polyacrylamide gels. Proteins were detected by silver staining.

2.8. Northern blots and dot blots:

RNA samples were prepared by extracting total RNA from plants using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen) as described above. Samples were then

heated at 55°C for 15 minutes, mixed with loading buffer (contains formamide, 5X MOPS, formaldehyde, glycerol, bromophenol blue), and electrophoresed on agarose gels containing 2M formaldehyde in 1X MOPS buffer (200mM MOPS pH 7.0, 80mM sodium acetate, 10mM EDTA, adjusted to pH8.0). Following electrophoresis, the gels were soaked in 10X SSC (1.5M sodium chloride, 150mM trisodium citrate, adjusted to pH 7.0) and transferred to nylon membranes. Membranes were baked at 80°C under vacuum for two hours. Prehybridization was carried out in 5mL of pre-warmed ExpressHyb solution (BD Biosciences) for 30 minutes at 68°C with continuous shaking. Hybridization was performed by adding the probe and incubating the membranes at 55°C for 1 hour with continuous shaking. RNAs that have been internally radiolabeled with [α-^{32}P]GTP (Perkin-Elmer) were used as RNA probes in the hybridization step. Membranes were then washed four times in wash solution 1 (4X SSC, 0.05% SDS) at room temperature and twice in wash solution 2 (0.1X SSC, 0.1% SDS) at 50°C. Results were visualized by autoradiography.

2.9. Mass Spectrometry

Bands selected for analysis were excised from silver-stained SDS-PAGE gels, and sent to the Ottawa Institute of Systems Biology (OISB) for analysis by LC MS/MS.

2.10. Transfections

All the HepG2 and HeLa cell line transfections were performed using the LipofectamineLTX and PLUS reagents (Invitrogen) and following the manufacturer's standard protocol. The ratios used were 1μg plasmid DNA: 20pmol RNA : 6μl lipofectamine LTX : 1μl PLUS reagent.

Results

3.1. INTERACTION OF POTATO SPINDLE TUBER VIROID (PSTVd) WITH THE HOST DNA-DEPENDENT RNA POLYMERASE II (RNAP II)

3.1.1. PSTVd is capable of replicating in vivo in tomato plants

The following experiment was performed to confirm whether our particular variety of the tomato plant (*Lycopersicon esculentum*, Rutgers variety) is a suitable host for PSTVd infection and replication. To this end, tomato seeds were planted and grown for 2 weeks. At this point, the plantlets were mechanically inoculated with *in vitro*-synthesized PSTVd trimeric RNA of the (+) polarity (PSTVd(+)3X). The plants were allowed to grow for an additional 2 weeks, at which point the leaves were harvested and a total RNA extraction was performed. Following extraction, total RNA was subjected to RT-PCR. The RT step was carried out in the presence of primers that anneal to the central region of either the (+) or (–) strand of the PSTVd RNA genome (primers L1 and L2, respectively; please refer to Appendix B for primer sequences). Amplification of the cDNA product using both of these primers (L1+L2) should yield a 180bp DNA product corresponding to the left half of the PSTVd genome, if PSTVd RNA is present in the RNA extract. Agarose gel electrophoresis was used to analyze products of the RT-PCR reactions (see Fig. 9). The RNA extract from healthy tomato leaves shows no band at 180bp. In contrast, the

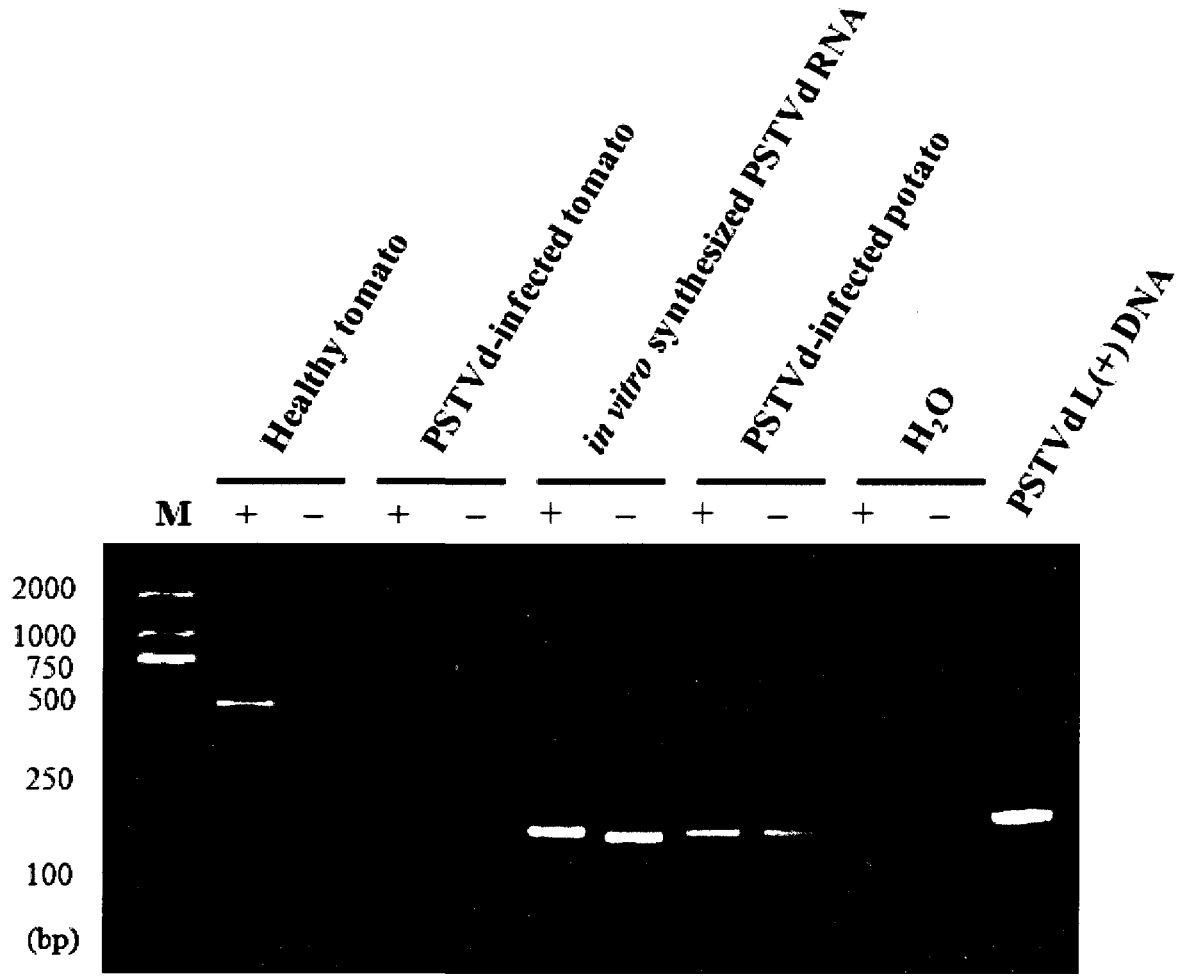

Healthy tomato
PSTVd-infected tomato
in vitro synthesized PSTVd RNA
PSTVd-infected potato
H2O
PSTVd L(+) DNA
M
+ − + − + − + − + −
2000
1000
750
500
250
100
(bp)

Fig. 9. Replication of PSTVd in tomato plants. 2-week old plantlets were inoculated with *in vitro*-synthesized PSTVd RNA of (+) polarity. Two weeks later total RNA from the leaves was isolated and subjected to RT-PCR using primers L1 and L2, which anneal to the central region of either the (+) or (–) strand PSTVd RNA, respectively. The expected RT-PCR product size is 180bp and corresponds to the left half of the PSTVd sequence. RT-PCR was performed on healthy and infected plants grown under the same conditions. As positive controls, *in vitro* synthesized PSTVd RNA (mix of both polarities) and total RNA isolated from PSTVd-infected potatoes (obtained from Agriculture Canada) were used.

RT-PCR products obtained from the RNA extracts of PSTVd-inoculated plants show a distinct band of the expected size. Both polarities of PSTVd were detected in the inoculated plants by this method. Our positive controls (*in vitro*-synthesized PSTVd(+) and PSTVd(–) RNAs, and an RNA extract obtained from PSTVd-infected potatoes (Agriculture Canada)) also show a 180bp product. These results indicate that PSTVd is capable of replicating in our tomato plants. Consequently, the Rudgers tomato will be used as the PSTVd host plant and as a source of host factors for all of our following experiments. Furthermore, this experiment shows that our method of mechanical inoculation is suitable. Several products at ~500bp were obtained in all of the reactions using tomato RNA extracts. These products will be interpreted in the discussion section.

3.1.2. Host RNAP II interacts specifically with the PSTVd RNA genome

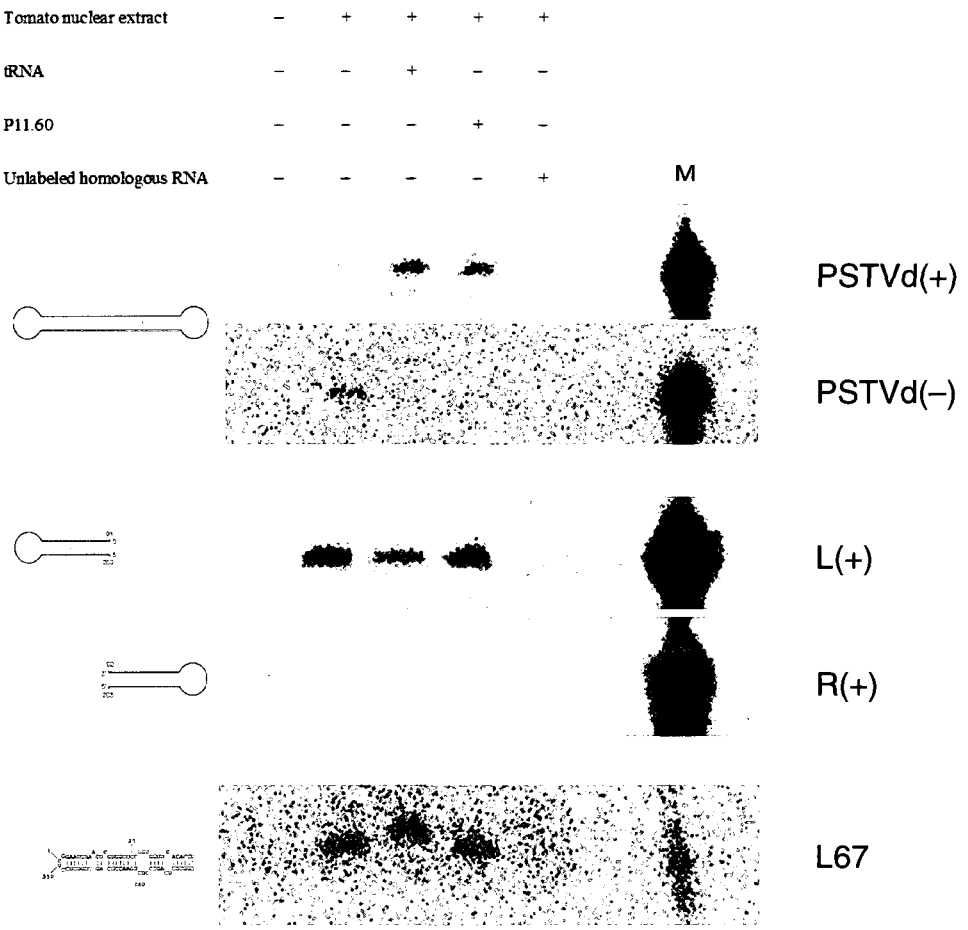
To determine whether host RNAP II interacts with PSTVd RNA, co-immunoprecipitation experiments using radiolabeled RNA and tomato nuclear extract (NE) as source of RNAP II were performed. Following incubation of PSTVd RNA with tomato NE, which allowed for the formation of RNA-protein complexes, mouse monoclonal antibody (mAb) H14, which recognizes the C-terminal domain of the largest subunit of RNAP II, was added. The samples were immunoprecipitated using protein-G-agarose beads. The immunoprecipitated complexes were resolved by denaturing polyacrylamide gel electrophoreses, and radiolabeled RNA was analyzed by autoradiography or phosphor imaging. The first co-immunoprecipitation experiment was conducted using full-length PSTVd RNA of (+) polarity. Under our

experimental conditions, a band that corresponds to radiolabeled PSTVd RNA was detected in the sample that was immunoprecipitated in the presence of tomato NE (see Fig. 10A, top panel). This band was not detectable in the sample that was immunoprecipitated in the absence of tomato NE. Two non-specific RNA competitors, namely tRNA and P11.60, were used in order to assess the specificity of interaction between PSTVd and RNAP II. When a 50-fold excess of tRNA, an unrelated RNA species, was added to the sample, no significant decrease in the amount of co-immunoprecipitated RNA was observed. To assess whether the interaction was caused by non-specific affinity of RNAP II for double-stranded RNAs, a small RNA hairpin, P11.60, derived from the peach latent mosaic viroid (PLMVd) (Pelchat and Perrault, 2004), was used as competitor. Upon the addition of a 50-fold excess of P11.60, a band corresponding to radiolabeled PSTVd RNA was still observed. However, when a 50-fold excess of non-radiolabeled homologous RNA was added, the band could no longer be detected.

The next co-immunoprecipitation experiment was performed with full-length PSTVd RNA of the (–) polarity. As in the previous experiment, a band corresponding to radiolabeled RNA was detected when tomato NE was added to the samples. However, this band was very faint and could only be detected when the image was overexposed. The bands were absent when either tomato NE was excluded, or when a 50-fold excess of non-specific competitors or homologous RNA were used.

These results provide direct evidence that RNAP II from tomato NE interacts specifically with PSTVd(+) RNA. The interaction with PSTVd(–), on the other hand, appears to be weak and non-specific.

A


Tomato nuclear extract – + + + +
tRNA – – + – –
P11.60 – – – + –
Unlabeled homologous RNA – – – – +
M
PSTVd(+)
PSTVd(–)
L(+)
R(+)
L67

B

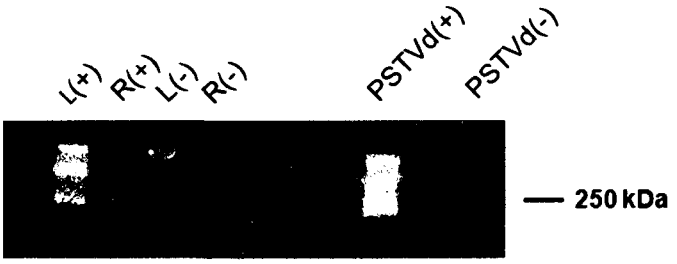

L(+)
R(+)
L(-)
R(-)
PSTVd(+)
PSTVd(-)
250 kDa

Fig. 10. Interaction of tomato RNAP II with the PSTVd RNA genome. (A) Co-immunoprecipitation experiments were carried out using linear full-length, [γ-^{32}P]ATP end-labeled PSTVd RNA of (+) polarity (first panel from the top) and (–) polarity (second from the top) and RNAP II from tomato NE. Experiments were also performed with various PSTVd-derived RNAs: left and right halves of the positive polarity of PSTVd (L(+) and R(+)), as well as the left terminal stem-loop domain of PSTVd(+) (L67). Results were obtained by running immunoprecipitates on 5% polyacrylamide gel, and visualized by phosphor imaging. A 50-fold excess of either tRNA or P11.60 was used as a non-specific competitor, and 50-fold excess of non-radiolabeled homologous RNA was used as a specific competitor. One tenth of the RNAs used in each co-immunoprecipitation are used as size markers. (B) Interactions were verified by RNA affinity chromatography using PSTVd-derived RNAs, followed by Western blotting against RNAP II. 200pmol RNA were oxidized and attached to adipic acid dihydrazide agarose beads and incubated with tomato NE. Unbound proteins were removed by centrifugation. Bound proteins were eluted, subjected to SDS-PAGE and Western blotting with anti-RNAP II antibody.

3.1.3. RNAP II interacts with the left terminal domain of PSTVd(+)

In order to narrow down the region of PSTVd with which RNAP II interacts, deletion analyses were performed. First, the PSTVd RNA genome was divided into two domains: the right half (R), which corresponds to the region between nucleotides 92 and 269, and the left half (L), which corresponds to the region between nucleotides 268 and 91. Appropriate primers were designed for PCR amplification of these fragments, and the resulting products were used as templates for *in vitro* transcription. The resulting PSTVd-derived RNAs were radiolabeled and used in co-immunoprecipitation experiments. These experiments were carried out with PSTVd-derived RNAs of the (+) polarity, as this polarity was found to interact with RNAP II. When the experiment was performed using RNA that corresponds to the right half of the PSTVd genome (R(+)), no RNA was found to co-immunoprecipitate with RNAP II. However, when the same experiment was performed using RNA that corresponds to the left half of PSTVd (L(+)), a band corresponding to radiolabeled L(+) RNA was observed (see Fig. 10.A, R(+) and L(+)). The addition of a 50-fold excess of a non-specific competitor (tRNA) did not decrease the amount of immunoprecipitated RNA. However, upon the addition of a 50-fold excess of non-radiolabeled L(+) RNA, the band was no longer detectable. The band corresponding to radiolabeled L(+) RNA was also absent in the negative control, in which no tomato NE was added.

The association of PNAP II with PSTVd L(+) RNA was verified by RNA affinity chromatography using the PSTVd-derived RNAs discussed above, followed by Western blotting against RNAP II (see Fig. 10B). 200pmol of each RNA species were oxidized and attached to adipic acid dihydrazide agarose beads and incubated with tomato NE. Unbound proteins were removed by centrifugation. Bound proteins were eluted, subjected to SDS-PAGE and Western

blotting with the mouse monoclonal anti-RNAP II antibody H14. A band at 220kDa corresponding to the largest subunit of RNAP II was visible in the PSTVd(+) and L(+) lanes. (The two additional bands that appear to be of higher molecular weight are most likely seen due to proteins that did not migrate into the gel, but were trapped in the well and between the stacking and resolving layers). No RNAP II was detected in cases where the other PSTVd-derived RNAs were tested (R(+), L(–), R(–) and PSTVd(–)).

Since only the left half of the (+) polarity of PSTVd appears to exhibit specific interaction, we decided to focus on this area in an attempt to locate the RNAP II interaction site. Truncation of this RNA region revealed that the left terminal segment consisting of a 67 nucleotide hairpin loop (L67) is still capable of specific interaction with RNAP II (see Fig. 10A, bottom panel).

3.1.4. Characterization of the putative PSTVd RNAP II promoter

After determining that the left terminal domain of PSTVd contains an RNA segment recognized by the host RNAP II, we decided to further investigate the features of this region. To this end, a bioinformatics approach was used. Manual alignment of 147 variants of PSTVd revealed that the left terminal domain of the viroid's genome is capable of adopting two distinct conformations: an unbranched rod-like structure, and a bifurcated structure (see Figs. 11 and 12) This possibility of a second structure arises due to a two-fold complementary sequence repeat present in the genome. Hence, the left terminal domain has a potential of forming base-pairing interactions in one of two ways, giving rise to two possible secondary structures of similar free

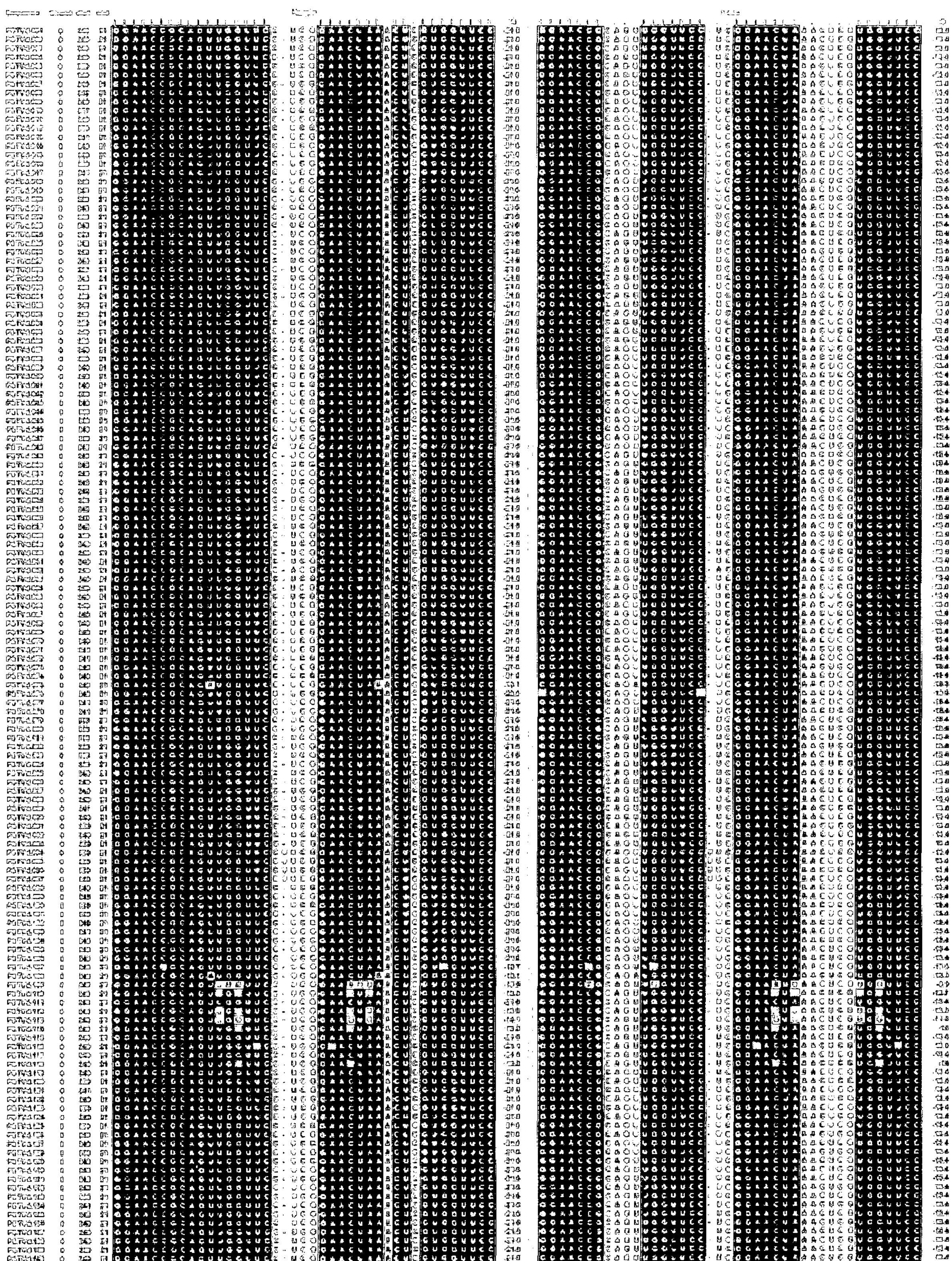


Fig. 11. Manual sequence alignment of the left terminal domain of PSTVd. 147 variants of PSTVd were compared. Two possible secondary structures (hairpin and the sub-optimal Y-like conformation), and their respective ΔG values are given for each of the sequences. (Alignment was performed by Martin Pelchat, and the ΔG values were calculated using RNAeval software from the ViennaRNA suite).

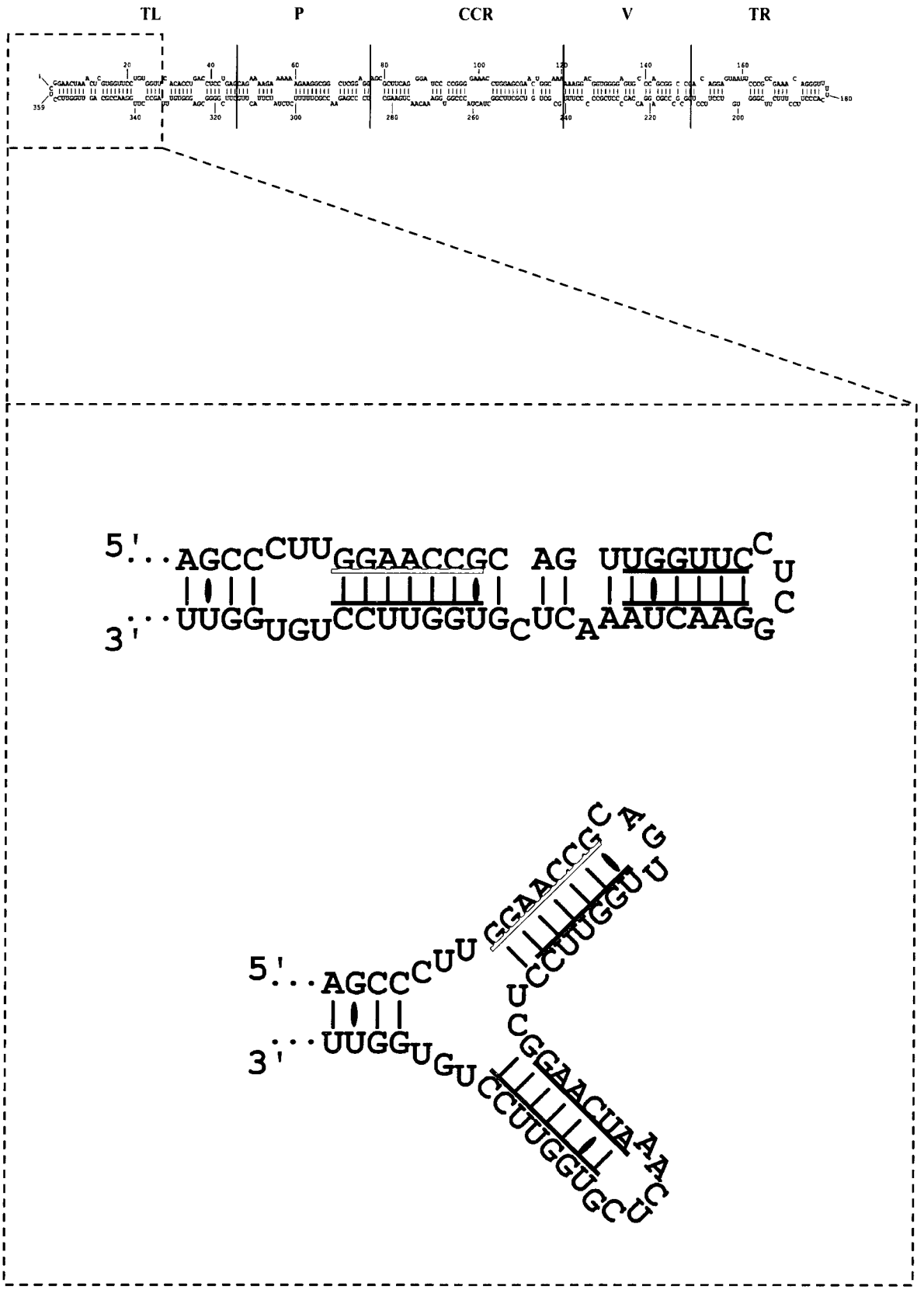

TL
P
CCR
V
TR
5'..AGCCCUUGGAACCGC AG UUGGUUCCU
3'..UUGGUGUCCUUGGUGCUCAAAUCAAGGC
5'..AGCCCUU
3'..UUGGUGUCCUUGGUGCU

Fig. 12. Location of the terminal left domain of PSTVd, and the two possible secondary structures it can adopt (the unbranched and the bifurcated forms). The different coloured lines are used to denote the four short nucleotide stretches that participate in the formation of both the unbranched rod-like conformation and the bifurcated form.

energies. In contrast, the right terminal domain is not capable of adopting the bifurcated structure, as it lacks this sequence feature.

To investigate the sequence and secondary structure requirements for interaction with RNAP II, various mutants of the left terminal 67 nucleotide region (L67) RNA were constructed. We were particularly interested in the importance of the bifurcated structure, as this feature was proposed only for the region that interacts with RNAP II. The same co-immunoprecipitation procedure used previously was employed in order to assess which of the mutants interact with RNAP II. In all, 21 mutants in addition to the wild-type sequence were generated and tested (see Fig. 13). Mutants that were constructed to have short stretches of base-pairing inverted in such a way as to preserve base-pairing that favours the formation of the rod-like structure (mutants L67-1a and L67-1b) or a mutant that still allows the possibility of forming both structures (L67-3) displayed specific interaction with RNAP II. However, those mutants constructed to favour the bifurcated form (mutants L67-2a and L67-2b) did not appear to interact with RNAP II.

In one study, Dingley and colleagues produced two PSTVd point-mutants which favour the formation of either the rod-like or the bifurcated form (Dingley *et al.*, 2003). We decided to test the effect of these two point-mutations for RNAP II binding. Consistent with the other results, the point-mutant favouring formation of the rod shape (mutant L67-U18C,A344G) exhibited specific interaction with RNAP II, while the mutation favouring the branched structure (mutant L67-U18C,A5G) did not show binding.

Additional co-immunoprecipitations were performed in order to further assess the importance of secondary structure. Mutants where various portions of base-pairing were disrupted (mutants L67-5a, b, c, d, e and f) did not display binding. Also, mutants where one of the two bulging nucleotides is removed (Fig. 13 cont'd; mutants L67-ΔA10 and L67-ΔC13),

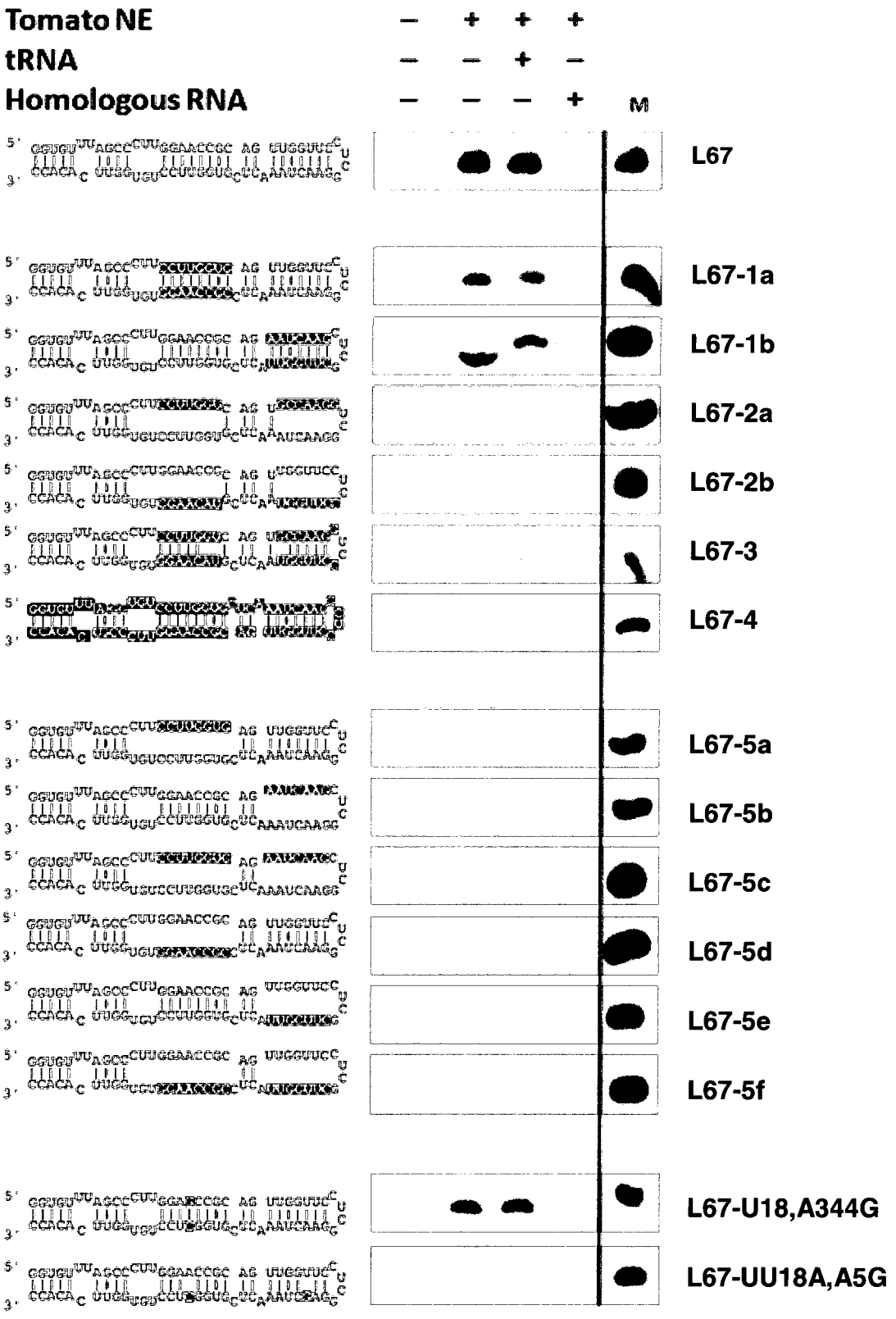

Tomato NE
tRNA
Homologous RNA
– + + +
– – + –
– – – +
M
L67
L67-1a
L67-1b
L67-2a
L67-2b
L67-3
L67-4
L67-5a
L67-5b
L67-5c
L67-5d
L67-5e
L67-5f
L67-U18,A344G
L67-UU18A,A5G

Fig. 13. Interaction of RNAP II with various mutants of the PSTVd left terminal domain. Co-immunoprecipitation experiments were performed as previously described using radiolabeled PSTVd L67 mutant RNA, tomato NE, and the H14 clone of the mouse monoclonal anti-RNAP II antibody. Results were obtained by running immunoprecipitates on 10% polyacrylamide gel, and visualized by autoradiography. A 50-fold excess of either tRNA was used as a non-specific competitor, and 50-fold excess of non-radiolabeled homologous RNA was used as a specific competitor. One tenth of the RNA used in each co-immunoprecipitation is used as a size marker. Inverted fonts and red crosses indicate changes made to the original sequence.

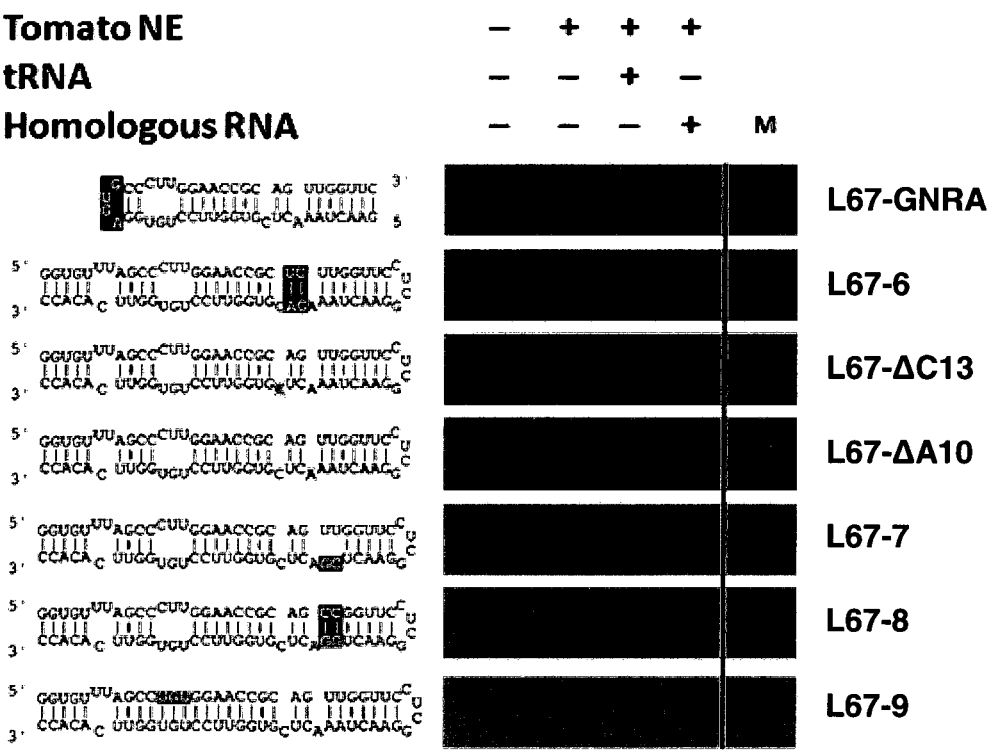

Tomato NE
tRNA
Homologous RNA
– + + +
– – + –
– – – + M
L67-GNRA
L67-6
L67-ΔC13
L67-ΔA10
L67-7
L67-8
L67-9

Fig. 13. (cont'd) Interaction of RNAP II with various mutants of the PSTVd left terminal domain.

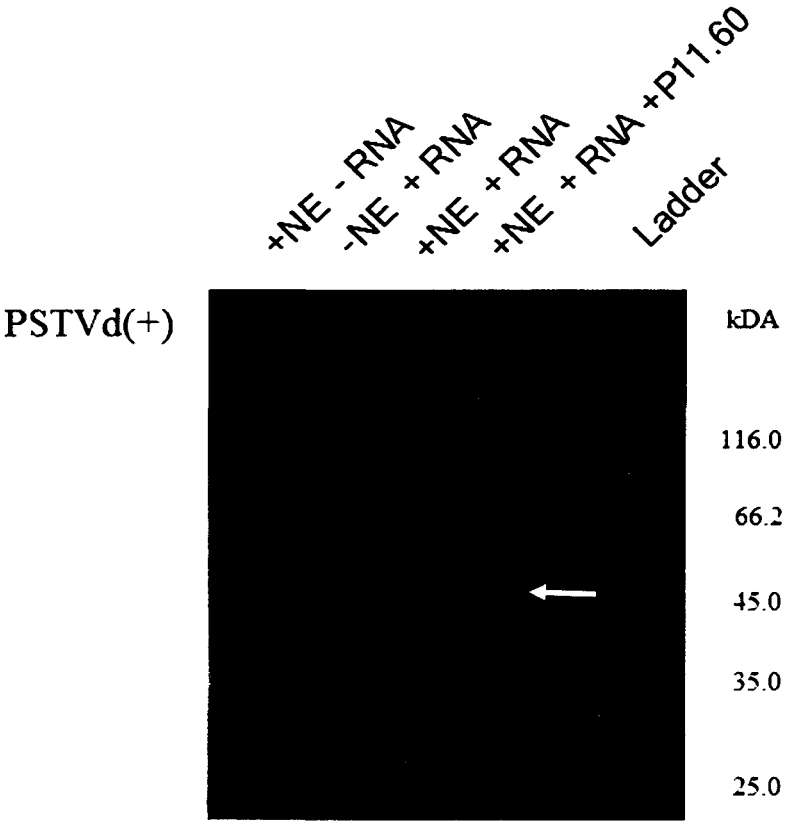
where the two strands of the entire RNA region are flipped (mutant L67-4), a mutant having the internal loop closed (mutant L67-9), and a mutant whose terminal loop has been removed and replaced by a GNRA loop on the opposite end (mutant L67-GNRA) did not exhibit interaction with RNAP II. One of our mutants, which had only two base-pairs disrupted (mutant L67-7) shows up as a very faint band following co-immunoprecipitation. A mutant which had a short (2 bp) region flipped (mutant L67-6), and a mutant having two consecutive A-U base pairs exchanged for two G-C base pairs (mutant L67-8) still displayed interaction with RNAP II.

Overall, these results suggest that the important features of this unconventional putative RNAP II promoter present on the PSTVd genome include a very specific RNA secondary structure. Our experiments show that only those mutants that are predicted to form a rod-like secondary structure, and whose internal loop and two additional bulging unpaired bases are not disrupted, are recognized by the RNAP II.

3.2. EVOLUTIONARY RELATIONSHIP BETWEEN THE HEPATITIS *DELTA* VIRUS (HDV) AND PLANT VIROIDS

3.2.1. Interaction of PSTVd and HDV with several shared proteins from their respective hosts

In addition to their interaction with a host polymerase, viroids rely on other host factors to accomplish the various steps associated with their replication, processing, and systemic movement in their host plant. One of our goals was to attempt to identify some of these factors. To this end, we used RNA affinity chromatography followed by mass spectrometry analyses. In brief, RNA of interest was oxidized and immobilized onto an adipic acid dihydrazide agarose bead matrix, and incubated with a tomato NE. Following incubation, the reactions were washed and unbound proteins removed. Bound proteins were eluted and resolved by SDS-PAGE (a representative gel is shown in Fig. 14). Those proteins that were found to bind specifically to PSTVd RNA were excised from the gel and identified by mass spectrometry. We were only successful in identifying two noteworthy proteins using this method: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and elongation factor 1-alpha (eEF1A1). Although we did not further investigate the role of these proteins in the viroid's life cycle, we did observe that these two proteins also interact with HDV RNA (Sikora *et al.*, 2009). Co-immunoprecipitation experiments performed by other members of our lab reveal specific interaction between the putative right promoter of HDV (R199G) and GAPDH and eEF1A1 (see Fig. 15).

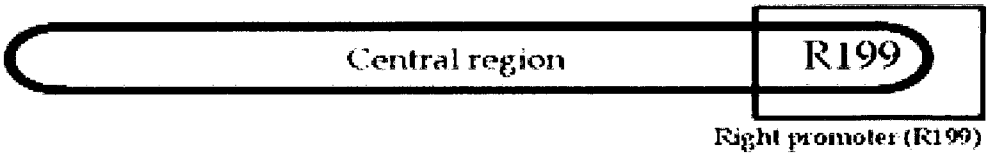


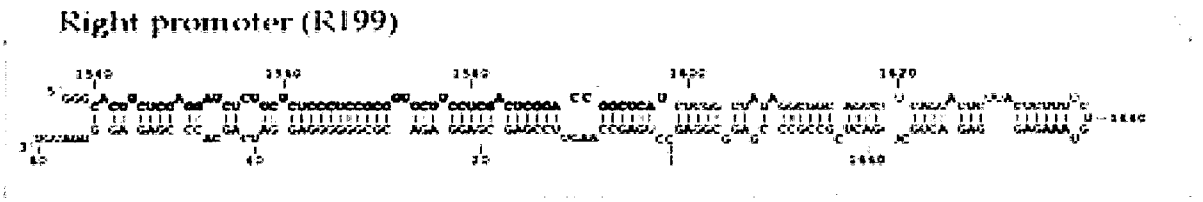
Proteins matching the same set of peptides:
gi|120665 Mass: 47954 Score: 234
Glyceraldehyde-3-phosphate dehydrogenase B,

gi|295810 Mass: 49599 Score: 53
elongation factor 1-alpha [Lycopersicon esculentum]

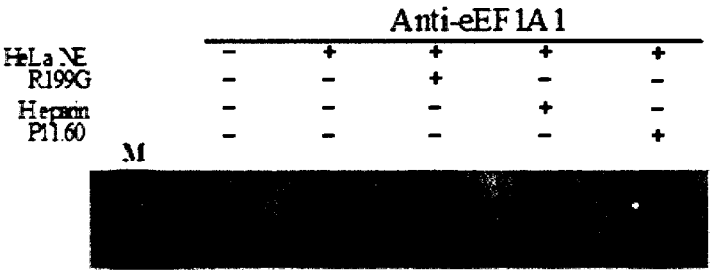
Fig. 14. Interaction of PSTVd(+) RNA with host proteins. Experiments were performed using full-length (+) strand PSTVd. Proteins were visualized by SDS-PAGE followed by silver staining, and were identified by mass spectroscopy. PSTVd-binding proteins are indicated by the white arrow.

A


Central region
R199
Right promoter (R199)


Right promoter (R199)

B


Anti-eEF1A1
HeLa NE
R199G
Heparin
P11.60
M

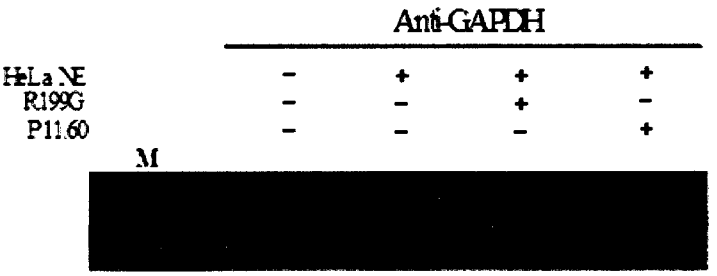

Anti-GAPDH
HeLa NE
R199G
P11.60
M

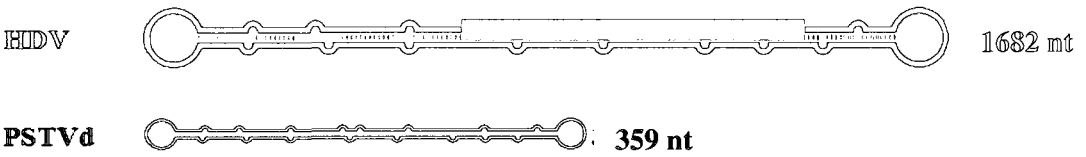
Fig. 15. Interaction of HDV with host proteins. (A) Schematic representation of the HDV RNA genome, including detailed section of the putative right promoter region (R199). The HDAg coding region is denoted by light blue shading. (B) Co-immunoprecipitations performed using R199G and either the eEF1A1 or GAPDH antibody. (Figure provided by Dorota Sikora)

3.2.2. HDV is capable of replicating in tomato plants

The previous experiments provide evidence that HDV and PSTVd may rely on common host factors to complete their respective life cycles, and therefore, may share common replication mechanisms. The next set of experiments was performed to determine whether HDV can employ proteins from a plant for its own replication, and cause infection in this new potential host.

For our first experiment, we inoculated 2-week old tomato plants with RNA corresponding to the dimer of the HDV genome (HDV(G)2X). More than unit-length RNA was used for this and the following experiments because it has been observed that tandem repeats of viroid sequences cause infection more effectively than monomers, since they are better processed into monomeric circular progeny viroids in the host. Monomeric viroid sequences, on the other hand, are non-infectious or only marginally infectious (Diener, 1986). The inoculated plants, along with healthy and mock-infected controls were allowed to grow for an additional 15 days. Fig. 16 shows a photograph taken 15 days post-inoculation. The HDV(G)2X-inoculated tomato plant displays significantly stunted growth when compared to the healthy plant grown in the same conditions. Our next aim was to assess the infection by RT-PCR. To this end, total RNA extracts were prepared from plant leaves and RT-PCR was performed as described in the previous section. The primers used for this experiment result in the amplification of the entire HDV RNA genome. Hence, a band at ~1700bp is expected if HDV is present in the RNA extract. Fig. 17A shows a band of the appropriate size present in the RT-PCR products obtained with the HDV(G)2X-inoculated plant RNA extracts. Both the genome and the antigenome of HDV were detected. In addition, the DNAs from these bands were cloned and sequenced, showing that they were derived from HDV. Our experiments also suggest that HDV was capable

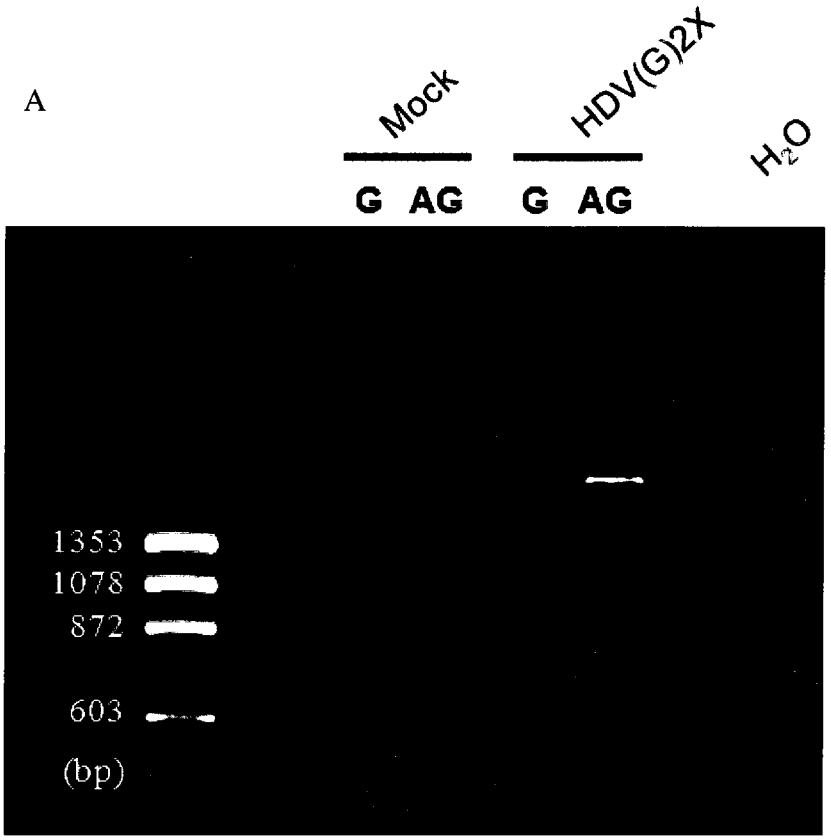
A



B

Fig. 16. (A) Comparison of the size and structure of HDV and PSTVd. (B) HDV infection in tomatoes. 20pmol of RNA corresponding to the dimer of the HDV genome (HDV(G)2X) was used to inoculate 2-week old tomato plants (left). The photograph was taken 15 days post-inoculation.

A


Mock
HDV(G)2X
H2O
G AG G AG
1353
1078
872
603
(bp)

B

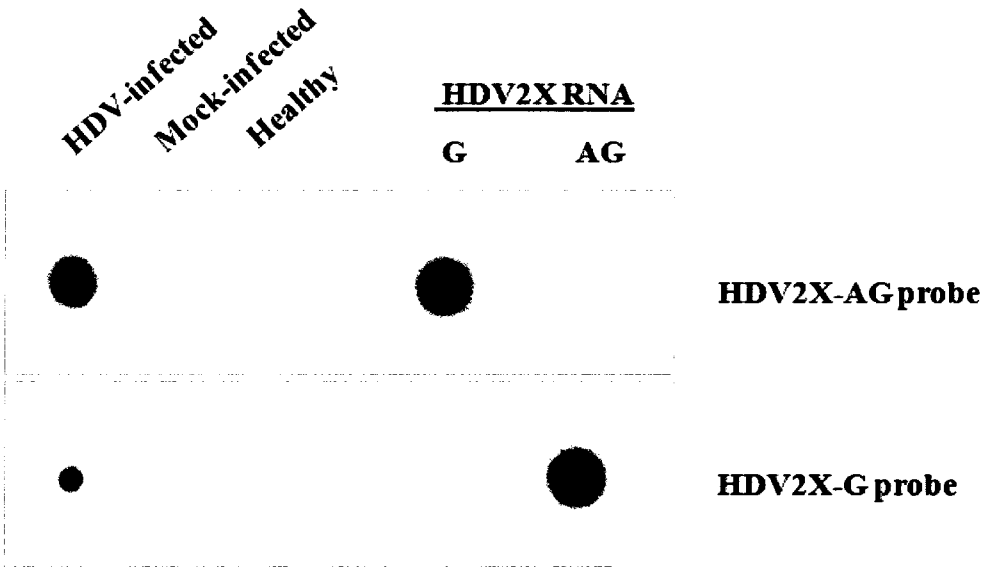

HDV-infected
Mock-infected
Healthy
HDV2X RNA
G AG
HDV2X-AG probe
HDV2X-G probe

Fig. 17. Replication of HDV in tomato plants. (A) 2-week old plantlets were inoculated with *in vitro*-synthesized genomic HDV dimer RNA (HDV(G)2X). Two weeks later total RNA from the leaves was isolated and subjected to RT-PCR using primers that anneal to either genomic or antigenomic HDV RNA, and yield an expected RT-PCR product of ~1700bp. (B) Dot blot assays were used to confirm the results. Total RNA isolated from infected, healthy, or mock-infected plants was blotted onto nylon membranes and hybridized with [α-^{32}P]GTP internally labeled probes that anneal to either genomic (HDV(AG)2X probe) or antigenomic (HDV(G)2X probe) HDV RNA. Genomic and antigenomic HDV2X RNAs were used as strand-specificity controls.

of spreading in the tomato plants, since the leaf that was initially inoculated with HDV(G)2X was removed prior to preparation of the RNA extract.

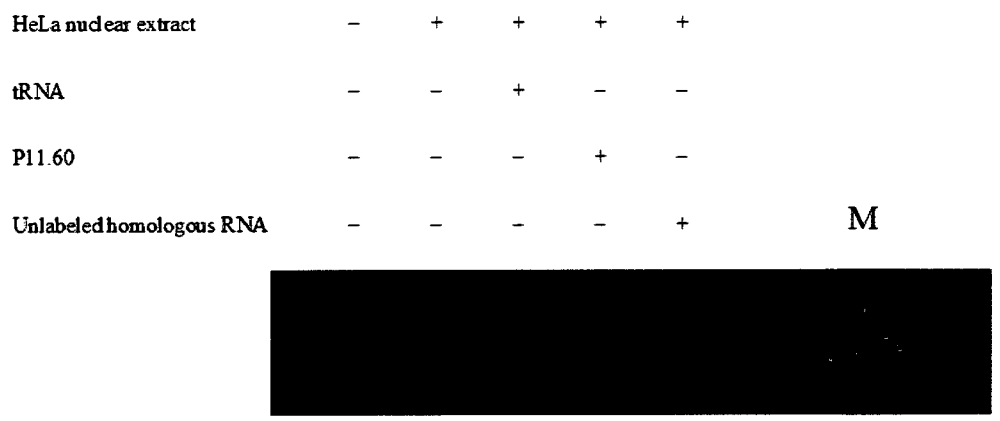
To confirm our findings, we repeated the inoculation experiments and assessed HDV accumulation by dot-blot analysis. For this experiment, RNA extracts, along with *in vitro*-synthesized HDV(G)2X and HDV(AG)2X strand specificity controls, were immobilized onto nylon membranes. Hybridization was carried out using either genomic or antigenomic radiolabeled dimeric HDV RNA probes. RNA extracts obtained from HDV(G)2X-inoculated plants were shown to contain genomic and antigenomic HDV RNA (Fig. 17B). In the healthy and mock-infected plant extracts, on the other hand, no HDV RNA was detected. The *in vitro*-synthesized HDV(G)2X and HDV(AG)2X controls on the right demonstrate that the hybridizations were strand-specific.

Together, this set of experiments provides strong evidence that HDV is capable of replicating in tomatoes, as well as spreading and inducing disease symptoms in the plant.

3.2.3. Presence of the small delta antigen (HDAg-S) is required for the replication of PSTVd in human cells

After establishing that HDV can replicate in a plant host, we wanted to test whether the reverse is also true (*i.e.* whether a plant viroid can replicate in a human host). First, we performed a co-immunoprecipitation using PSTVd(+) RNA, a HeLa nuclear extract (HeLa NE), and an anti-RNAP II monoclonal antibody (clone 8WG16). We observed that PSTVd(+) RNA does, in fact, interact with the human RNAP II (see Fig. 18A). The intensity of the band

A


HeLa nuclear extract – + + + +
tRNA – – + – –
P11.60 – – – + –
Unlabeled homologous RNA – – – – +
M

B

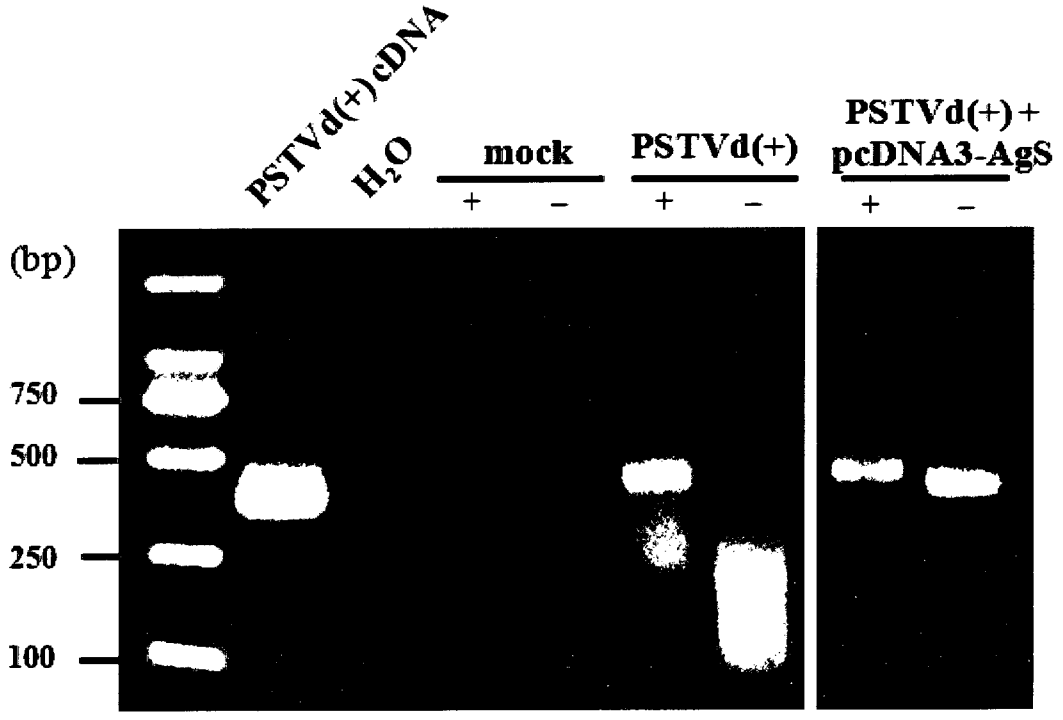

PSTVd(+) cDNA
H2O
mock
PSTVd(+)
PSTVd(+) + pcDNA3-AgS
+ – + – + –
(bp)
750
500
250
100

Fig. 18. Replication of PSTVd in human cells. (A) Co-immunoprecipitation experiments were carried out using full-length, [γ-^{32}P]ATP end-labeled PSTVd(+) RNA and HeLa NE. The 8WG16 clone of the mouse monoclonal anti-RNAP II antibody was used. Results were obtained by running immunoprecipitates on 5% polyacrylamide gel, and visualized by autoradiography. A 50-fold excess of either tRNA or P11.60 was used as a non-specific competitor, and 50-fold excess of non-radiolabeled homologous RNA was used as a specific competitor. One tenth of the RNAs used in each co-immunoprecipitation are used as size markers. (B) Total RNA was extracted from HepG2 cells that have been transfected with PSTVd(+)3X RNA, PSTVd(+)3X RNA + HDAg-S expressing plasmid, or mock reansfected. The RNA was subjected to RT-PCR. A primer that anneals to either the (+) or (–) polarity of PSTVd was used in the RT step, and primers that amplify the full length of PSTVd were used in the PCR step. (Fig. 18B was contributed by Dorota Sikora)

corresponding to the immunoprecipitated RNA did not decrease upon addition of a 50-fold molecular excess of tRNA. However, the band appears to be somewhat weaker when a 50-fold molecular excess of P11.60 is used as competitor. Since P11.60 is a putative promoter sequence derived from PLMVd, perhaps it is not the most suitable non-specific competitor for this experiment.

Next, we decided to investigate the replication of PSTVd in human cells. To this end, human hepatocellular liver carcinoma (HepG2) cell line was transfected with a PSTVd trimer (PSTVd(+)3X) RNA. Replication of PSTVd was assessed by RT-PCR. To our surprise, only PSTVd(+) was present in the transfected cells (see Fig. 18B). We were unable to detect any PSTVd(–) RNA. It has been reported that the HDV-encoded HDAg-S is vital for HDV replication in humans. HDAg-S was proposed to bind RNAP II directly and stimulate transcription by displacing the negative elongation factor (**NELF**) (Yamaguchi et al., 2001). Therefore, the ability of HDV to express HDAg-S may allow it to infect a human host. PSTVd and other viroids, however, do not express any proteins. NELF, whose presence has not been shown in plants to date, may prevent viroids from replicating in human cells. In order to test this theory, we inoculated HepG2 cells with PSTVd(+)3X and a plasmid expressing HDAg-S (pcDNA3-Ag-S, see Fig. 18B). RT-PCR results showed that the co-transfected cells contained both PSTVd(+) and PSTVd(–) RNA. In addition, sequencing results show that the amplified DNA is, indeed, derived from PSTVd (not shown).

Overall, these experiments demonstrate that the presence HDAg-S is required for PSTVd replication in HepG2 cells.

3.2.4. An HDV mutant lacking the HDAg-coding region (miniHDV) can replicate in tomatoes

The previous experiments provide evidence that HDV can replicate in plants and that PSTVd can replicate in human cells if supplied with HDAg-S. If the role of HDAg-S in this case is to bind directly to RNAP II, thereby displacing NELF and stimulating transcription, then this protein should not be necessary for replication of PSTVd and HDV in plants, as they lack NELF. To test this hypothesis, we have constructed an HDV mutant that lacks the HDAg-coding region. We have named this mutant, which closely resembles a viroid in its structure and size, miniHDV (see Fig. 19). To examine replication of miniHDV in plants, we inoculated 2-week old tomato plants with an RNA corresponding to the miniHDV 1.5mer of the genomic polarity (miniHDV(G)1.5X), as previously described, and allowed them to grow for a further 2 weeks. At this point, the leaves were harvested and total RNA was extracted. Presence of miniHDV RNA was established by RT-PCR (see Fig. 20). We observed that the inoculated plants contained miniHDV of genomic and antigenomic polarity, as determined by the band at ~350 base pairs. In contrast, healthy plants contained no miniHDV RNA.

Next, the presence of miniHDV RNA was confirmed by Northern blot analysis. Radiolabeled miniHDV monomer RNAs of either genomic or antigenomic polarity (miniHDV(G) or miniHDV(AG)) were used as hybridization probes. We were able to detect both genomic (Fig. 21A) and antigenomic (Fig. 21B) miniHDV in the RNA extracts obtained from the inoculated plants. In contrast, miniHDV RNA was absent from the extracts obtained from healthy and mock-infected plants. Several bands are visible in the miniHDV-infected lanes, which may correspond to the different forms of miniHDV RNA (i.e. monomeric, multimeric and circular RNA).

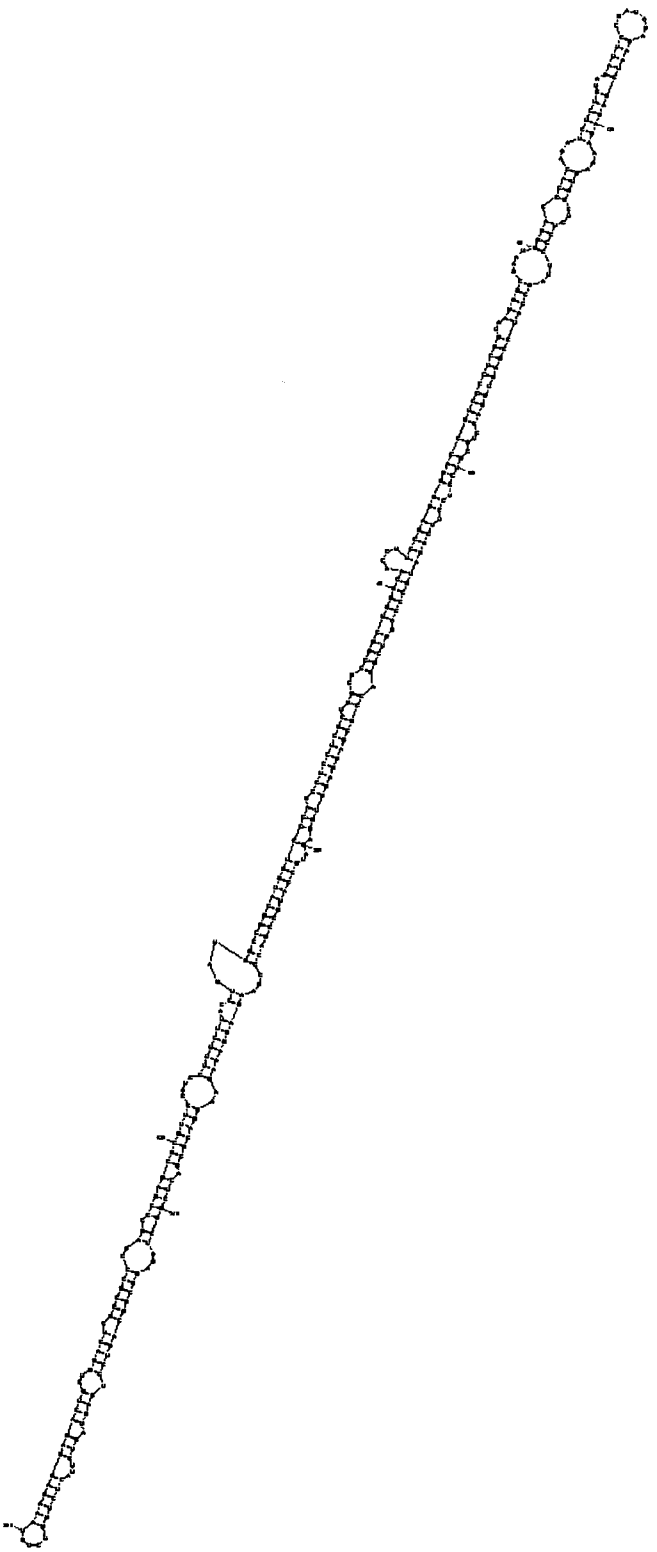


Fig. 19. Schematic representation of the miniHDV RNA genome. Secondary structure prediction was performed using the *mfold* web server for nucleic acid folding (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1-2.3.cgi).

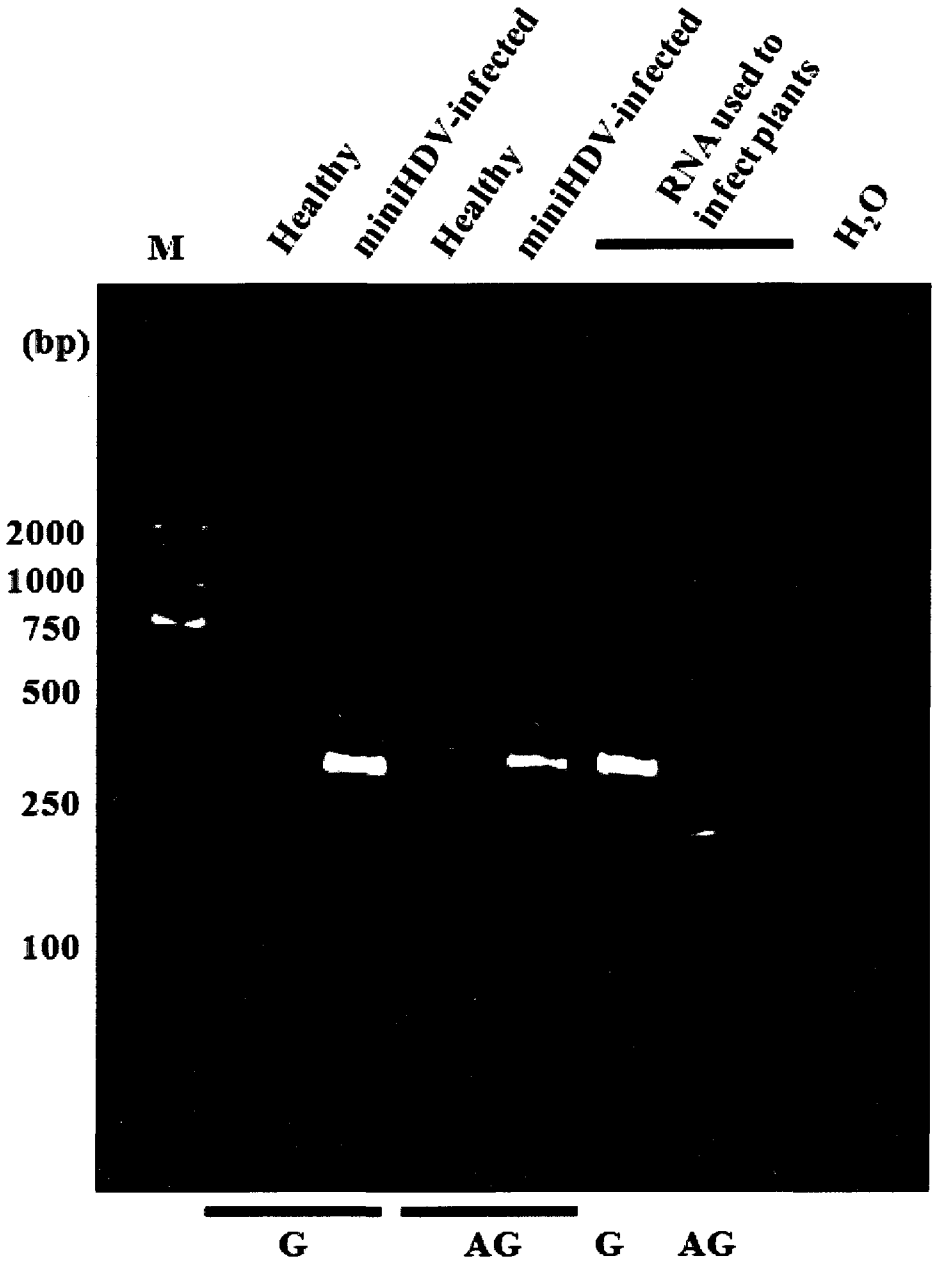
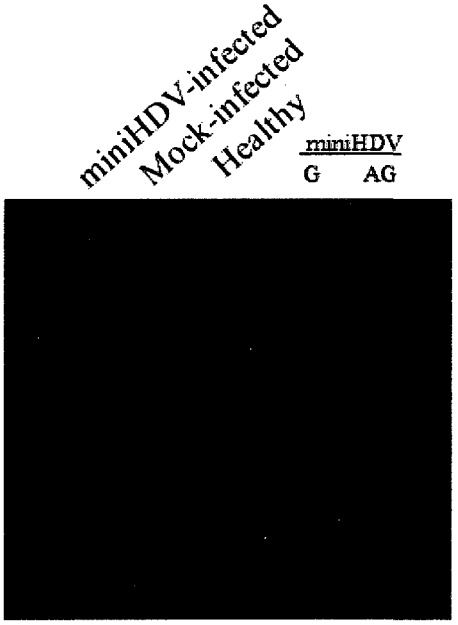

M
Healthy
miniHDV-infected
Healthy
miniHDV-infected
RNA used to infect plants
H2O
(bp)
2000
1000
750
500
250
100
G
AG
G
AG

Fig. 20. Replication of miniHDV in tomatoes. 2-week old tomato plants were inoculated with miniHDV(G)1.5X RNA. 2 weeks later, total RNA was extracted and subjected to RT-PCR analysis, along with total RNA extracted from healthy plants. Primers used in the RT step anneal to either the genomic or the antigenomic polarity of miniHDV. The PCR step results in the amplification of the entire miniHDV genome (342nt long). RT-PCR was also performed on the miniHDV(G)1.5X RNA used to infect the plants.


miniHDV-infected
Mock-infected
Healthy
miniHDV
G
AG

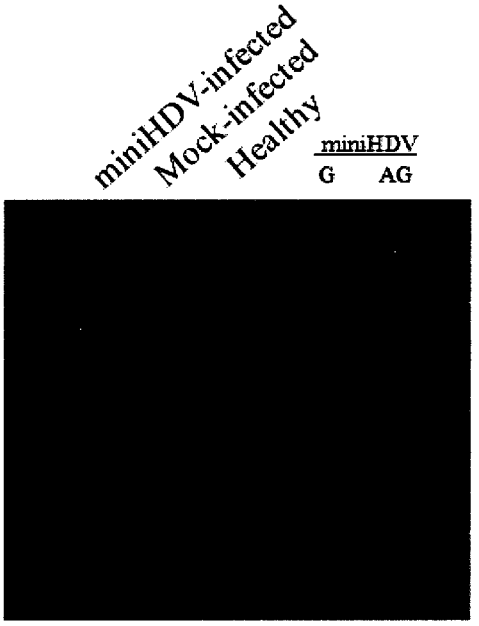

miniHDV-infected
Mock-infected
Healthy
miniHDV
G
AG

Fig. 21. Northern blot analysis of miniHDV replication in tomatoes. Total RNA extracts from miniHDV-infected, healthy, or mock-infected plants were prepared as described above. Following electrophoresis and transfer of RNA onto nylon membranes, hybridization was carried out in the presence of [α-^{32}P]GTP internally labelled RNA probes that anneal to either genomic (miniHDV(AG)1.5X probe, left panel) or antigenomic (miniHDV(G)1.5X probe, right panel) miniHDV RNA. *In vitro* synthesized miniHDV(G) and miniHDV(AG) RNAs were used as strand-specificity controls.

3.2.5. miniHDV can replicate in HeLa cells only if supplied with HDAg-S

Finally, we investigated the replication of miniHDV in human cells. For this study, HeLa cells were transfected with: miniHDV(G)1.5X RNA, miniHDV(G)1.5X RNA + HDAg-S-expressing plasmid, or the HDAg-S-expressing plasmid alone. Replication was assessed by RT-PCR analysis of the HeLa RNA extracts. The results of this experiment are displayed in Fig. 22. Under our experimental conditions, we were able to detect both polarities of miniHDV only in those cells transfected with both miniHDV(G)1.5X RNA and the plasmid that expresses HDAg-S. In those cells transfected with miniHDV(G)1.5X RNA alone, only genomic miniHDV was detected. No miniHDV was detected in the cells transfected with the HDAg-S-expressing plasmid alone.

Overall, this experiment provides evidence that miniHDV requires the presence of HDAg to be able to replicate in HeLa cells.

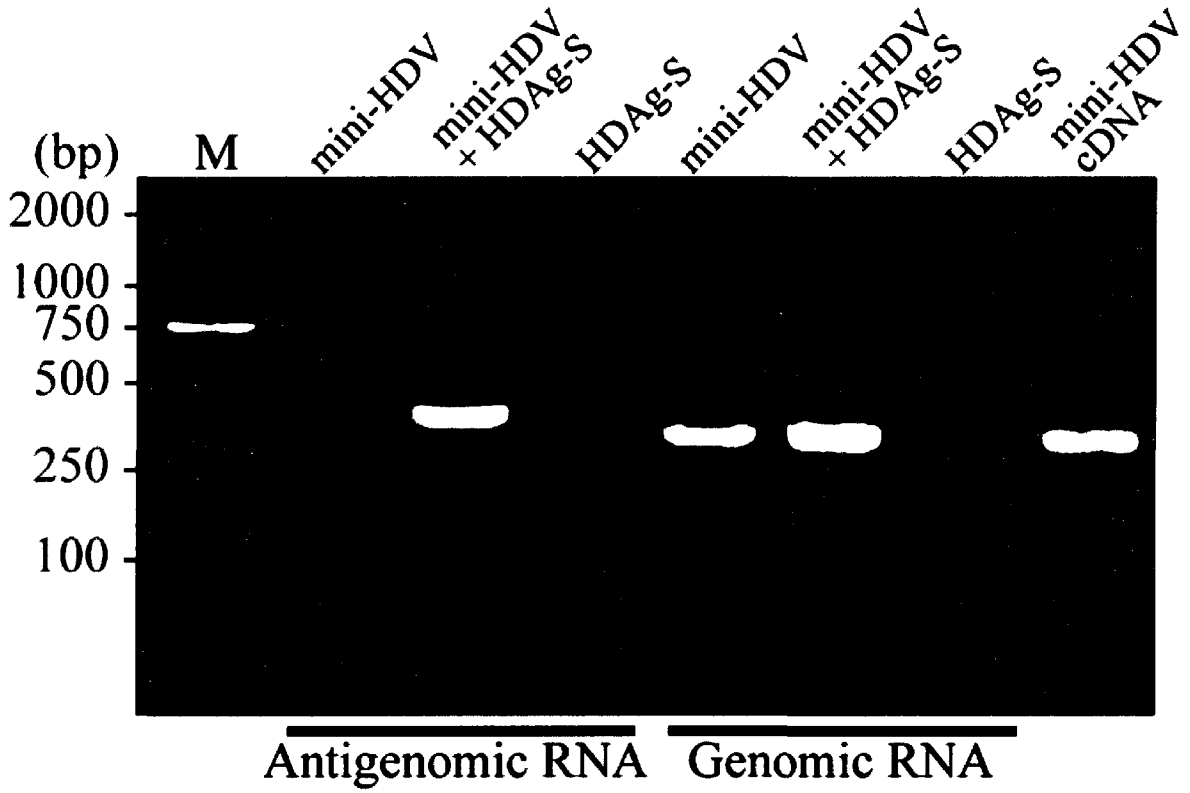

(bp)
M
mini-HDV
mini-HDV + HDAg-S
HDAg-S
mini-HDV
mini-HDV + HDAg-S
HDAg-S
mini-HDV cDNA
2000
1000
750
500
250
100
Antigenomic RNA
Genomic RNA

Fig. 22. Replication of miniHDV in HeLa cells. Total RNA was extracted from HeLa cells that have been transfected with miniHDV(G)1.5X RNA, miniHDV(G)1.5X RNA + HDAg-S expressing plasmid, or the HDAg-S expressing plasmid alone. The RNA extracts were subjected to RT-PCR. A primer that anneals to either the (G) or (AG) polarity of miniHDV was used in the RT step, and primers that amplify the full length of miniHDV were used in the PCR step.

Discussion

4.1 INTERACTION OF PSTVd WITH THE HOST DNA-DEPENDENT RNA POLYMERASE II

In order to in investigate the interaction of PSTVd RNA with any of its host's proteins, it was first necessary to find a suitable host in which PSTVd may replicate. For this, we chose the tomato (*Lycopersicon esculentum)*, as it has previously been used in PSTVd studies. To test whether our particular variety (Rutgers) is a suitable host, we infected the plants with a trimeric PSTVd RNA of the (+) polarity and assessed replication by RT-PCR. We were able to detect both (+) and (–) polarity PSTVd RNA in the infected plants, indicating that replication did occur (see Fig. 9). Therefore, this variety of the tomato was used as a host plant, and a source of host proteins in all of our future experiments. Interestingly, our RT-PCR results show an additional product of higher-than-expected size in the first four lanes. Since this product was present only in the tomato samples, regardless of whether they have been infected or not, we interpret this band to be the result of primers annealing to a nucleic acid present in tomatoes. This is a likely explanation since it has been reported that viroids display some sequence similarity to certain cellular RNAs (Hashimoto and Koganezawa, 1987; Diener, 1981; Haas *et al.*, 1988). In fact, this may be one of the ways in which they exert their pathogenic effects. In the future, it would be of

interest to further explore the identity of this cellular nucleic acid, as it may provide clues about interactions of viroids with host nucleic acids and potential viroid pathogenesis mechanisms.

RNA synthesis is a highly conserved process that can be divided into three distinct steps: initiation, elongation and termination. Initiation of transcription occurs once an RNA polymerase (RNAP) has located and bound to its promoter sequence, thus creating a "closed complex". Therefore, the association of RNAPs should indicate sites of transcription (Smale and Kadonaga, 2003). In this study, the first step of PSTVd RNA transcription by RNAP II (*i.e.* promoter recognition) was investigated. To this end, co-immunoprecipitation experiments using tomato nuclear protein extract (NE) and PSTVd-derived RNAs were performed with the goal of understanding more about the interaction of RNAP II with PSTVd, and locating the putative RNAP II promoter on the PSTVd genome. What makes this case somewhat unusual is the fact that RNAP II is typically associated with RNA transcription from a DNA template. Unlike animals, plants do possess RNA-dependent RNA polymerases, but to date there is no evidence that they are involved in the life-cycle of viroids. Instead, several studies have linked the host RNAP II to the replication of *Pospiviroids* (Schindler and Mühlbach, 1992; Warrilow and Symons, 1999).

First, we performed co-immunoprecipitations using full-length radiolabeled PSTVd RNAs of both polarities, tomato NE as source of RNAP II, and a monoclonal antibody raised against RNAP II (clone H14). When the experiment was performed with PSTVd(+), a band corresponding to the precipitated RNA can be seen in the lane containing the NE, whereas no band is visible in the lane where no NE was added to the reaction column (top panel, Fig. 10A). This result suggests that RNAP II from the tomato NE interacts with PSTVd(+) RNA. To test the specificity of this interaction, several reactions using specific and non-specific competitors were

performed. When a 50-fold molecular excess of a non-specific competitor was added to the samples, no decrease in the amount of immunoprecipitated RNA was observed. Two separate non-specific competitors, tRNA and P11.60, were used. P11.60 is a short RNA hairpin loop fragment derived from peach latent mosaic viroid (PLMVd). This RNA species has been shown previously to be a promoter for the *E. coli* RNAP (Pelchat *et al.*, 2002), making it an ideal competitor for our experiment. Since we were able to precipitate PSTVd(+) RNA in the presence of an excess of either of these competitors, we conclude that the reaction between RNAP II and PSTVd(+) is a specific one. This implies that RNAP II does not bind to RNAs in general, but preferentially interacts with the PSTVd RNA. Interestingly, an increase in the band intensity can be observed for the lanes containing a non-specific competitor when compared to the lane with NE alone. Our interpretation for this observation is that there are many RNA-binding proteins present in the NE. By saturating these proteins with an unrelated RNA species, more PSTVd RNA is available for interaction with RNAP II. However, this observation should be taken with caution, as our co-immunoprecipitation experiment is not quantitative. Finally, we examined the interaction in the presence of a specific competitor, in this case homologous non-radiolabeled RNA. When a 50-fold molecular excess of non-radiolabeled PSTVd(+) RNA was added to the reaction, the PSTVd(+) band was no longer detectable, as expected. Overall, this experiment supports the idea that RNAP II interacts with PSTVd(+) in a specific manner.

We obtained surprising results when the same co-immunoprecipitation experiment was performed with the full-length PSTVd RNA of the (–) polarity. A faint band could be observed in the lane corresponding to the reaction containing NE when the phosphor screen was overexposed. However, no bands were noticeable for the lanes corresponding to the samples containing a competitor. There are several explanations for this. First, it is possible that, because

the manner in which we synthesize the RNA results in a linear molecule, the nick is located exactly at the region that is recognized by RNAP II. However, this is unlikely. Our PSTVd(–) RNA molecule is open between nucleotides 359 and 1. We performed additional experiments using a PSTVd-derived RNA species that corresponds to the left half of the genome, and does not contain a nick between nucleotides 359 and 1 (see Fig. S.1, Appendix C). This RNA also did not interact with RNAP II. Alternatively, RNAP II may only interact weakly or non-specifically with PSTVd(–). If both the (+) and the (–) polarity of PSTVd were replicated by the same polymerase, one would expect this polymerase to have a stronger affinity for the (–) strand because this is the less abundant strand *in vivo*. This leaves the possibility that host polymerases other that RNAP II may be involved in the life-cycle of PSTVd. Although this is a surprising observation, involvement of additional polymerases in the replication of other subviral pathogens has been reported. It was previously believed that the replication of HDV was performed exclusively by RNAP II. However, a recent study has reported the association of both RNAP I and RNAP III with HDV-derived RNAs (Greco-Stewart *et al.*, 2009).

Once we have established specific interaction between RNAP II and PSTVd(+), we used deletion analyses in order to narrow down the RNA region to which RNAP II binds. Here, we divided the PSTVd genome into the left and right halves, and synthesized RNAs corresponding to these regions. Interactions between these PSTVd-derived RNAs and RNAP II from tomato NE were investigated by co-immunoprecipitations, as previously. Using this approach, we have determined that RNAP II interacts with the left half of the PSTVd(+) strand RNA, but not with the right half. Once again, competition experiments have shown these interactions to be specific. By truncating the L(+) region, we observed that an RNA corresponding to the left terminal 67 nucleotides of PSTVd(+), termed L67, was still capable of interaction with RNAP II, suggesting

that the putative RNAP II promoter may be located within this hairpin loop region. Overall, these experiments support the hypothesis that RNAP II binds the PSTVd(+) RNA genome, and is responsible for generation of the (–) strand RNA. Based on our results, the replication of PSTVd most likely starts at a defined site, located within the left terminal domain of the genome, rather than randomly. However, since we only looked at interaction, but not activity, we could not conclude that the binding of RNAP II to this RNA fragment leads to RNA transcription. Nonetheless, this finding is in accordance with results of other experiments aimed at locating the transcription start site for PSTVd. More specifically, in a study by Kolonko and colleagues, it has been reported that the transcription of PSTVd starts in the left terminal loop (Kolonko *et al.*, 2006). In this study, the initiation site of transcription from the (+) strand circles to the (–) strand replication intermediates was analyzed by adding circular (+) strand PSTVd molecules to potato NE prepared from non-infected culture cells. After allowing transcription to occur, the *de novo* synthesized (–) strands were purified by affinity chromatography, and their 5'-ends were determined by primer extension analysis. The results of the study indicate a single transcription start site, either nucleotide C1 or U359, located in the left terminal loop. α-amanitin was used to verify the involvement of RNAP II from the potato NE. However, this study examined only the generation of (–) strands from a (+) template. Transcription of the (+) strand from a (–) template and the determination of its start site was not investigated.

In a recent study, Zhong and colleagues attempted to identify specific motifs of PSTVd necessary for systemic trafficking and replication (Zhong *et al.*, 2008). A genome-wide mutational analysis of PSTVd and infection of *Nicotiana benthamiana* was used to assess the importance of various loops and bulges present in the viroid's genome. Four loop/bulge motifs that were found to be essential for PSTVd replication are located in the left terminal domain.

Three additional motifs were found in the central region. All other loops/bulges were associated with viroid trafficking. These findings are consistent with our results. It is possible that the transcriptional promoter is located in the left terminal domain, while the motifs located in the central region are critical for later events in replication, possibly via the interaction/recruitment of unknown host factors.

Overall, our findings suggest that only the left terminal region of the (+) polarity serves as a suitable RNAP II interaction site. This implies that the region in question contains a special sequence or structural feature distinguishing it as a possible RNAP II promoter. Upon closer inspection by manual alignment of 147 PSTVd variants, the left terminal domain was shown to have the possibility of adopting one of two conformations of similar free energy, a rod-like and a bifurcated form, due to a two-fold complementary sequence repeat (see Fig. 12). This feature is unique to the left terminal domain. In a previous study, Dingley and colleagues examined the structure of the 69nt region of the left terminal domain of PSTVd and established, via NMR and thermodynamic analysis, that the elongated rod-like form is favoured under native conditions (Dingley *et al.*, 2003). However, another study relying on structure prediction calculations and nuclease and chemical probing has concluded that the bifurcated form is adopted as the native conformation (Gast *et al.*, 1996).

With the goal of characterizing the putative RNAP II promoter and identifying the sequence/structural requirements necessary for interaction with RNAP II, we designed a series of PSTVd left terminal 67 nucleotide (L67) mutants. To investigate the importance of the two possible conformations in RNAP II-interacting recognition, special focus was given to designing mutants that eliminate the possibility of forming one of the two conformations. Co-immunoprecipitation experiments performed with these mutants consistently showed that in

addition to the wild-type sequence (L67), those mutants that favour the formation of the elongated rod-like structure (Fig. 13, mutants L67-1a, L67-1b and L67-3) interact with RNAP II, whereas those that favour the bifurcated form do not (mutants L67-2a and L67-2b). In addition, the two point mutants that stabilize either the rod-like structure (mutant L67-U18C,A344G) or the bifurcated structure (mutant L67-U18C,A5G) were examined. These mutants were previously shown by NMR to truly adopt their predicted conformation (Dingley *et al.*, 2003). Once again, only the mutant favouring rod-like formation co-immunoprecipitated with RNAP II. Together, these results suggest that the elongated rod-like conformation plays a vital role in mediating the interaction with RNAP II, while the bifurcated form does not. Although the bifurcated form does not seem to be relevant for the initial interaction, it may serve an important biological purpose for another step in the viroid's replication cycle, since this feature appears to be conserved among the *Pospiviroidae* family members (Dingley *et al.*, 2003). There are several examples of the same RNA domain of a viroid genome being able to adopt different conformations under different conditions (reviewed by Tabler and Tsagris, 2004; Zhong *et al.*, 2006). It is precisely this conformation switching that may be essential for diverse functions. Further study is necessary to determine whether this is the case with the left terminal domain of PSTVd. Perhaps RNA affinity chromatography could be used to identify additional proteins that bind the left terminal region of PSTVD. It would be interesting to compare which proteins interact with the wild-type RNA and which interact with point-mutants that favour one of the two conformations.

Our results provide evidence that RNAP II likely recognizes a specific secondary structure, rather than sequence of this unusual putative promoter. We conclude this based on the observation that interaction occurred even when short stretches of sequence were flipped or

altered (Fig. 13, mutants L67-1a, L67-1b, L67-3, L67-6 and L67-8). Additional co-immunoprecipitations were performed in order to further assess the importance of secondary structure. Mutants where portions of base-pairing were disrupted (mutants L67-5a, b, c, d, e and f) did not display binding. This indicates the importance of base-pairing and double-strandedness of the RNA. However, this does not seem to be sufficient since several other mutants, predicted to contain only minor changes in the overall secondary structure, did not demonstrate interaction. For example, mutants where one of the two bulging nucleotides is removed (mutants L67-ΔA10 and L67-ΔC13), where the two strands of the whole RNA region are flipped (mutant L67-4), a mutant having the internal loop closed (mutant L67-9), and a mutant whose terminal loop has been removed and replaced by a GNRA loop on the other end (mutant L67-GNRA) did not exhibit interaction with RNAP II. This suggests that the overall secondary structure, together with some very specific features such as internal loops and bulges, comprises the features vital for interaction. Upon altering any of these key features, binding was no longer observed. One of our mutants, which has two base-pairs disrupted (mutant L67-7) shows up as a very faint band following co-immunoprecipitation. This indicates that this mutant may be only weakly binding. Unfortunately, our co-immunoprecipitation experiment is not quantitative, and hence, we cannot assess the degree or strength of interaction. It would be interesting to determine quantitatively the binding affinity of RNAP II for each of the mutants, but direct binding experiments would be challenging due to the difficulty of purifying the functional multi-subunit RNAP II. Another limitation of our approach is the fact that we cannot determine the nature of the interaction and all the events and factors that mediate it. Likely, RNAP II is not binding directly to the RNA, but rather it may be recruited via other host factors acting as transcription factors, which remain to be identified.

Although promoters for RNAP II transcription typically contain a TATA-element, an initiator sequence (INR), or both, promoters lacking these elements have been described (Novina and Roy, 1996). The exact events leading to the formation of the transcriptional complex or initiation of transcription remain unclear. PSTVd does not contain a TATA-element or an INR, making it an appealing model for the study of transcriptional mechanisms of these unconventional promoters. The results of the present study provide evidence that specific features of the rod-like secondary structure of PSTVd may be recognized and accepted by RNAP II. Numerous previous studies have shown that other hairpin structures, such as those found in the terminal regions of PSTVd, can be recognized by polymerases. For example, studies with avocado sunblotch viroid (ASBVd), the type member of the *Avsunviroidae* family, have revealed that transcription is initiated at a hairpin motif, located in the terminal loop (Navarro and Flores, 2000). In this case, transcription is believed to be determined by secondary structure, rather than the RNA sequence itself. In addition, a study with peach latent mosaic viroid (PLMVd), another *Avsunviroidae* family member, has demonstrated that *E.coli* RNAP binds the terminal end of the viroid molecule and transcribes PLMVd-derived templates (Pelchat and Perrault, 2005). As in the ASBVd study, the enzyme in this case is thought to recognize the particular secondary structure of the molecule, since mutations that disrupt the rod-like conformation interfere with transcription. Apart from viroids, transcription initiation within terminal loops has recently been characterized for the hepatitis *delta* virus (HDV) RNA genome (Greco-Stewart *et al.*, 2007). HDV is a ~1700 nucleotide long subviral satellite RNA virus of the hepatitis B virus. It shares several key features with viroids; it is a single-stranded, circular RNA molecule that adopts an unbranched rod-like secondary structure and replicates via a rolling-circle mechanism. In the above-mentioned study, co-immunoprecipitation experiments have revealed that human RNAP II

interacts specifically with HDV-derived RNAs at sites corresponding to the two terminal stem-loop regions of both polarities of HDV. In addition, analyses of these regions show a strong preference for maintaining the rod-like conformation.

In several of the above-mentioned studies it has been demonstrated that the same domains on the genomic and the antigenomic strand act as the promoter region. This further lends support to the hypothesis that specific secondary structures, rather than the RNA sequences, are recognized by the polymerase. Unfortunately, we were unable to determine whether a polymerase binds the same PSTVd-derived RNA region on the (–) strand as on the (+) strand. The previously mentioned study by Kolonko and colleagues in which the transcription start site of PSTVd was attributed to the left terminal loop only examined the generation of (–) strand replicative intermediates from (+) strand circles (Kolonko *et al.*, 2006). Whether or not the same region is also the start site for the synthesis of (+) strands from a (–) strand template is unknown.

It is well known that *Pospiviroidae* family members contain several characteristic structural domains. Pospiviroids may possess either a terminal conserved hairpin (TCH) or a terminal conserved region (TCR), both of which are located within the terminal left domain of the viroid's genome, and share a high degree of homology among the different family members. Although these regions are highly conserved, their function still remains unknown. Our experiments suggest that these regions could, in fact, serve as the RNA promoter for a DNA-dependent RNA polymerase. Unfortunately, not much effort has been undertaken yet in characterizing the promoters of other *Pospiviroidae* members. Such studies could be useful in determining common promoter features that may have been conserved throughout this family of viroids.

4.2 EVOLUTIONARY RELATIONSHIP BETWEEN THE HEPATITIS *DELTA* VIRUS (HDV) AND PLANT VIROIDS

Apart from being the smallest pathogen known to infect humans, the hepatitis *delta* virus (HDV) has several other features which distinguish it from other viruses. In fact, this satellite virus of HBV shares many more common characteristics with plant viroids than it does with other viruses. Both HDV and viroids are single-stranded circular RNA molecules that are replicated by a host polymerase via a rolling-circle mechanism. HDV shares common characteristics with both major families of viroids. For instance, similarly to *Pospiviroidae*, the HDV RNA genome adopts an unbranched, rod-like secondary structure and is replicated in the host cell's nucleus, most likely by RNAP II. Similarly to *Avsunviroidae*, HDV follows the symmetric pathway of the rolling-circle replication mechanism, and processes replication intermediates by self-cleavage via an intrinsic ribozyme motif. However, despite these parallels, a notable difference exists between HDV and viroids. Whereas viroids have no coding capacity, HDV possesses an open reading frame that encodes for two proteins, the large (HDAg-L) and small (HDAg-S) antigens. These proteins are identical except for the 19 additional amino acids present at the C-terminus of HDAg-L, resulting from an RNA-editing event of the termination codon of the HDAg-S gene. Both proteins serve a distinct purpose in the HDV life-cycle (Kuo *et al.*, 1989; Chang *et al.* 1991; Chao *et al.*, 1990). An intriguing explanation exists concerning the similarities of these subviral RNA pathogens. It has been proposed that HDV has evolved from an ancient viroid-like RNA element through the capture of a cellular transcript, thus enabling it to acquire a new host. Although our knowledge of the evolutionary origin of HDV is still limited, several studies lend

support to the above hypothesis. Previous studies have identified a host gene whose protein product interacts with HDAg, thereby affecting the replication of HDV. DNA sequence analysis of this protein, termed *delta*-interacting protein A (DIPA), have revealed that it is a cellular homologue of HDAg (Brazas and Ganem, 1996).

If HDV and viroids do share a common evolutionary origin, then perhaps they still rely on common mechanisms and host factors for their propagation. As mentioned above, it is already known that both pathogens use the same rolling circle replication strategy. In the present study we have explored the possibility that both HDV and PSTVd may interact with several common host factors. Aside from RNAP II, both PSTVd and HDV may interact with the host glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and elongation factor 1-*alpha* (eEF1A1), as suggested by the co-immunoprecipitation and affinity chromatography analyses presented in Figs. 14 and 15B. Both GAPDH and eEF1A1 have been shown to be RNA-interacting proteins, although their functions in the life-cycle of HDV and PSTVd are beyond the scope of this study. Interestingly, GAPDH is a key glycolytic enzyme, although numerous non-glycolytic activities such as helicase activity, interaction with nucleic acids and stimulation of RNAP II transcription have been reported (Karpel and Buchard, 1981; Morgenegg *et al.*, 1986).

Since HDV and PSTVd appear to rely on common host factors, we decided to examine how replication is affected when their respective hosts are switched. First, we looked at replication of HDV RNA in tomato plants. Using both RT-PCR and dot-blot analysis, the presence of both genomic and antigenomic HDV RNA was confirmed in plants inoculated with the genomic HDV RNA dimer. In addition, HDV also appears to be able to spread in the host plant. This was concluded based on the presence of HDV RNA of both polarities in the RNA extracts prepared following removal of the leaf that was initially inoculated. This suggests that

HDV was capable of replicating and spreading effectively in the plant host. We also assessed the replication of PSTVd in human cells. First we performed a co-immunoprecititation experiment using PSTVd(+) RNA, HeLa nuclear extract (NE) and a monoclonal antibody raised against the C-terminal domain of the largest subunit of RNAP II. The results of this experiment indicate that RNAP II present in the HeLa NE specifically interacts with PSTVd. However, the interaction between PSTVd and human RNAP II may be weaker or less specific than the viroid's interaction with tomato RNAP II. This can be concluded based on the observation that upon addition of the competitor P11.60, the intensity of the band corresponding to PSTVd RNA decreased in the co-immunoprecipitation experiment using HeLa NE (see Fig. 18.A), whereas no decrease was observed when tomato NE was used (see Fig. 10A). Subsequently, we attempted to infect the human HepG2 cells with PSTVd. RT-PCR results showed that following inoculation with the trimeric PSTVd(+) RNA, no viroid RNA replication occurred. However, when the cells were co-infected with the same RNA in addition to a plasmid that expresses HDAg-S, replication of PSTVd RNA was observed. Hence, HDAg-S appears to play an important role in allowing the replication of PSTVd in human cells, just as it does for HDV. It has previously been shown that the role of HDAg in HDV transcription activation is to directly bind to RNAP II. Upon binding, HDAg displaces the cellular negative elongation factor (NELF), thereby stimulating HDV transcription (Yamaguchi *et al.*, 2001). NELF, which is a negative regulator of transcription elongation by RNAP II, has been found to share some sequence similarity to HDAg. Perhaps viroids can readily replicate in a plant host since plants lacks NELF. However, in a human host, NELF acts as a negative regulator of viroid RNA transcription. Just like for HDV replication, HDAg may displace NELF from host RNAP II and promote transcription of the viroid RNA. This evidence supports the idea that HDAg is an evolutionary adaptation of HDV that was

necessary for it to obtain a new host. To further explore this hypothesis, we have constructed an HDV mutant from which the HDAg-coding region has been removed (miniHDV). This HDV-derived mutant very much resembles a viroid in both size (342 nucleotides long) and structure (see Fig. 19). We attempted to infect both plants and human cells with miniHDV and assessed its replication in these two hosts. RT-PCR and Northern blot results show that miniHDV was indeed able to replicate in tomato plants, following inoculation with a miniHDV RNA 1.5mer of genomic polarity (miniHDV(G)1.5X). In addition, the Northern blots reveal presence of miniHDV RNAs of various sizes. We interpret these multiple bands as the various RNA replication intermediates (*i.e.* monomeric circular, monomeric linear, dimeric and multimeric RNAs). This further provides support that miniHDV is replicating, in the expected manner, in the tomato plants. However, no replication occurred in HeLa cells inoculated with miniHDV(G)1.5X. This is expected, since miniHDV no longer expresses the HDAg necessary for its replication. When HeLa cells were infected with both miniHDV(G)15.X and the HDAg-expressing plasmid, replication of miniHDV was observed.

Overall, these results indicate that HDAg-S is a crucial factor for the replication of both PSTVd and HDV in human cells. However, the presence of HDAg-S is not necessary for their replication in plants. Since plants do not express NELF, which negatively regulates RNAP II transcription, they do not require a factor to counteract its effect. If HDV did evolve from an ancient RNA element resembling present day viroids, it is tempting to suggest that the ability to express HDAg was an acquirement that made it possible for HDV to infect and replicate in humans.

Conclusion

Potato spindle tuber viroid (PSTVd) and the hepatitis *delta* virus (HDV) belong to a collection of small auto-replicating RNA pathogens, along with other viroids, satellite RNAs and viroid-like plant satellites. These infectious RNA molecules cause diverse diseases in plants and animals, and hence have a significant impact on the agriculture industry as well as health care. For this reason, it is of great value to gain a better understanding of the nature of these pathogens and the mechanisms by which they operate. In addition, this unique set of RNA pathogens may present an evolutionary link between present day life and the proposed ancient RNA world, and serve as an important model for the study of RNA form/function and RNA-DNA relationship.

Of particular interest is the elucidation of viroid replication mechanisms. This study focused on the interaction between PSTVd and the host DNA-dependent RNA polymerase II (RNAP II). We have demonstrated that RNAP II interacts with the left terminal domain of the viroid's RNA genome in a specific manner, which seems to involve key structural features present in this hairpin-loop region. This finding is in accordance with numerous other studies of subviral RNA pathogens where putative RNA promoters for a DNA-dependent polymerase were mapped onto terminal hairpin-loops. However, our current knowledge of viroid replication is still limited. It would be interesting to characterize and compare putative promoters of different viroids in greater depth, with the aim of finding common features. Future endevours should also include the elucidation of the detailed events, other host factors involved and their precise roles in the replication process.

The present study also examined the relationship between viroids and HDV, as these pathogens share key structural and functional characteristics. HDV and viroids appear to share common strategies and host factors to fulfill their respective life-cycles. We found that both HDV and an HDV mutant lacking the HDAg protein-coding region (miniHDV) can replicate in a plant host. However, miniHDV and PSTVd can replicate in human cells only in the presence of the small *delta* antigen (HDAg-S). Together, these results provide support for the hypothesis that HDV evolved from a viroid-like element through the capture of a cellular transcript necessary for its adaptation to a human host. As mentioned earlier, greater comprehension of HDV propagation including the roles of host factors, virus-encoded HDAg, as well as RNA elements are of great interest, since this pathogen is responsible for a significant amount of morbidity and mortality around the world.

Finally, it is critical that knowledge obtained from the study of both viroids and HDV be integrated for a greater overall understanding of the life-cycle of these unique subviral RNA pathogens.

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Contributions of Collaborators

All of the laboratory work was performed by Teodora Bojić, with the exception of the following. Dorota Sikora performed the co-immunoprecipitations of R199G with GAPDH and eEF1A1, presented in Figure 15B, as part of the publication "Sikora, D., Greco-Stewart, V.S., Miron, P. and Pelchat, M. (2009). The hepatitis delta virus RNA genome interacts with eEF1A1, p54(nrb), hnRNP-L, GAPDH and ASF/SF2. Virology. 390, 71-78." Dorota Sikora also performed the HepG2 infection experiments presented in Figure 18B. Martin Pelchat performed the sequence analysis presented in Figure 11. The RT-PCRs presented in Figures 20 and 22 were performed by Erica Schissel and Jen Yi, respectively.

Appendices

APPENDIX A – Genomic sequences (obtained from Subviral RNA database, at http://subviral.med.uottawa.ca)

PSTVd:
Potato spindle tuber viroid isolate M1, complete genome (359 nucleotides), accession number AF459005

```
  1 CGGAACTAAA CTCGTGGTTC CTGTGGTTCA CACCTGACCT CCTGACAAGA AAAGAAAAAA
 61 GAAGGCGGCT CGGAGGAGCG CTTCAGGGAT CCCCGGGGAA ACCTGGAGCG AACTGGCAAA
121 AAAGGACGGT GGGGAGTGCC CAGCGGCCGA CAGGAGTAAT TCCCGCCGAA ACAGGGTTTT
181 CACCCTTCCT TTCTTCGGGT GTCCTTCCTC GCGCCCGCAG GACCACCCCT CGCCCCCTTT
241 GCGCTGTCGC TTCGGCTACT ACCCGGTGGA AACAACTGAA GCTCCCGAGA ACCGCTTTTT
301 CTCTATCTTA CTTGCTTCGG GGCGAGGGTG TTTAGCCCTT GGAACCGCAG TTGGTTCCT
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HDV:
Hepatitis D virus genomic RNA, complete genome (1682 nucleotides), accession number X04451

```
   1 ATGAGCCAAG TTCCGAACAA GGATTCGCGG GGAGGATAGA TCAGCGCCCG AGAGGGGTGA
  61 GTCGGTAAAG AGCATTGGAA CGTCGGAGAT ACAACTCCCA AGAAGGAAAA AAGAGAAAGC
 121 AAGAAGCGGA TGAATTTCCC CATAACGCCA GTGAAACTCT AGGAAGGGGA AAGAGGGAAG
 181 GTGGAAGAGA AGGAGGCGGG CCTCCCGATC CGAGGGGCCC GGCGGCCAAG TTTGGAGGAC
 241 ACTCCGGCCC GAAGGGTTGA GAGTACCCCA GAGGGAGGAA GCCACACGGA GTAGAACAGA
 301 GAAATCACCT CCAGAGGACC CCTTCAGCGA ACAGAGAGCG CATCGCGAGA GGGAGTAGAC
 361 CATAGCGATA GGAGGGGATG CTAGGAGTTG GGGGAGACCG AAGCGAGGAG GAAAGCAAAG
 421 AGAGCAGCGG GGCTAGCAGG TGGGTGTTCC GCCCCCCGAG AGGGGACGAG TGAGGCTTAT
 481 CCCGGGGAAC TCGACTTATC GTCCCCACAT AGCAGACTCC CGGACCCCCT TTCAAAGTGA
 541 CCGAGGGGGG TGACTTTGAA CATTGGGGAC CAGTGGAGCC ATGGGATGCT CCTCCCGATT
 601 CCGCCCAAGC TCCTTCCCCC CAAGGGTCGC CCAGGAATGG CGGGACCCCA CTCTGCAGGG
 661 TCCGCGTTCC ATCCTTTCTT ACCTGATGGC CGGCATGGTC CCAGCCTCCT CGCTGGCGCC
 721 GGCTGGGCAA CATTCCGAGG GGACCGTCCC CTCGGTAATG GCGAATGGGA CCCACAAATC
```

781 TCTCTAGCTT CCCAGAGAGA AGCGAGAGAA AAGTGGCTCT CCCTTAGCCA TCCGAGTGGA
841 CGTGCGTCCT CCTTCGGATG CCCAGGTCGG ACCGCGAGGA GGTGGAGATG CCATGCCGAC
901 CCGAAGAGGA AAGAAGGACG CGAGACGCAA ACCTGCGAGT GGAAACCCGC TTTATTCACT
961 GGGGTCGACA ACTCTGGGGA GAGGAGGGAG GGTCGGCTGG GAAGAGTATA TCCTATGGGA
1021 ATCCCTGGCT TCCCCTTATG TCCAGTCCCT CCCCGGTCCG AGTAAAGGGG GACTCCGGGA
1081 CTCCTTGCAT GCTGGGGACG AAGCCGCCCC CGGGCGCTCC CCTCGTTCCA CCTTCGAGGG
1141 GGTTCACACC CCCAACCTGC GGGCCGGCTA TTCTTCTTTC CCTTCTCTCG TCTTCCTCGG
1201 TCAACCTCCT AAGTTCCTCT TCCTCCTCCT TGCTGAGGTT CTTTCCCCCC GCCGATAGCT
1261 GCTTTCTCTT GTTCTCGAGG GCCTTCCTTC GTCGGTGATC CTGCCTCTCC TTGTCGGTGA
1321 ATCCTCCCCT GGAAGGCCTC TTCCTAGGTC CGGAGTCTAC TTCCATCTGG TCCGTTCGGG
1381 CCCTCTTCGC CGGGGGAGCC CCCTCTCCAT CCTTATCTTT CTTTCCGAGA ATTCCTTTGA
1441 TGTTTCCCAG CCAGGGATGT TCATCCTCAA GTTTCTTGAT TTTCTTCTTA ACCTTCCGGA
1501 GGTCTCTCTC GAGTTCCTCT AACTTCTTTC TTCCGCTCAC CCACTGCTCG AGAACCTCTT
1561 CTCTCCCCCC GCGGTTTTTC CTTCCTTCGG GCCGGCTCAT CTTCGACTAG AGGCGACGGT
1621 CCTCAGTACT CTTACTCTTT TCTGTAAAGA GGAGACTGCT GGCCCTGTCG CCCAAGTTCG
1681 AG

miniHDV:

GGCCGGCATG GTCCCAGCCT CCTCGCTGGC GCCGGCTGGG CAACCTTCCG
AGGGGACCGT CCCCTCGGTA ATGGCGAATG GGACCCACAA ATCTCTCTAG
ATTCCGATAG AGAATCGAGA GAAAAGTGGC TCTCCCTTAG CCATCCGAGT
GGACCTGCGT CCTCCTTCGG ATGCCCAGGT CGGACCGCGA GGAGGTGGAG
ATGCCATGCC GACCCGAAGA GGAAAGAAGG ACGCGACTCG GCTAGAGGCG
GCAGTCCTCA GTACTCTTAC TCTTTTCTGT AAAGAGGAGA CTGCTGGACT
CGCCGCCCGA GCCCGAGCGC GTTCCATCCT TTCTTACCTG AT

APPENDIX B – Primer sequences (T7 promoter sequences are underlined)

1) Primers used for the amplification of full-length PSTVd of (–) polarity (primers 1 and 2), and (+) polarity (primers 3 and 4)

1:
5'GGAATTCTAATACGACTCACTATAGGGAACCAACTGCGGTTCCAAGGGCTAAA CACCC 3'

2:
5'GGAACTAAACTCGTGGTTCCTGTGGTTC 3'

3:
5'GGAACCAACTGCGGTTCCAAGGGCTAAACACCC 3'

4:
5'GGAATTCTAATACGACTCACTATAGGGAACTAAACTCGTGGTTCCTGTGGTTC 3'

2) Primers used for the amplification of the left half of PSTVd (primers L1 and L2 were used for generating the (–) polarity, and L3 and L4 were used for the (+) polarity)

L1:
5'GGAATTCTAATACGACTCACTATAGGGATCCCTGAAGCGCTCCTCCGAGCCGC 3'

L2:
5'GAAACAACTGAAGCTCCCGAGAACCGC 3'

L3:
5'GATCCCTGAAGCGCTCCTCCGAGCCGC 3'

L4:
5'GGAATTCTAATACGACTCACTATAGGGAAACAACTGAAGCTCCCGAGAACCGC 3'

3) Primers used for the amplification of the right half of PSTVd (primers R1 and R2 were used for generating the (–) polarity, and R3 and R4 were used for the (+) polarity)

R1:
5'GGAATTCTAATACGACTCACTATAGGGCCACCGGGTAGTAGCCGAAGCGACAG CGC 3'

R2:
5'CCCCGGGGAAACCTGGAGCGAACTGGC 3'

R3:
5'CCACCGGGTAGTAGCCGAAGCGACAGCGC 3'

R4:
5'GGAATTCTAATACGACTCACTATAGGGCCCCGGGGAAACCTGGAGCGAACTGG
C 3'

T7 primer:

5'TAATACGACTCACTATAGGG 3'

4) Oligos used for the synthesis of PSTVd left terminal domain mutants:

- **L67:** (wild-type sequence)
  5' CAGGTGTGAA CCACAGGAAC CACGAGTTTA GTTCCGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-1a:**
  5' CAGGTGTGAA CCACACCTTG GCGGAGTTTA GTTCCGAGGA
  ACCAACTCACCAAGGAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-1b:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTAAC CAAGCGAGCT
  TGATTCTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-2a:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTTTA GTTCCGACCT
  TGGCACTGAC CAAGGAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-2b:**
  5' CAGGTGTGAA CCACACCTTG ATCGAGTTAC CAAGGGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-3:**

5' CAGGTGTGAA CCACACCTTG ATCGAGTTAC CAAGGGACCT
TGGCACTGAC CAAGGAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-4:**
  5' ACAAATCGGG AACCTTGGCG TCAACCAAGG AGCCTTGATT
  TGAGCACCAA GGACACCAAG TGTGGACCCC TATAGTGAGT CGTATTA 3'

- **L67-5a:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTTTA GTTCCGAGGA
  ACCAACTCGC CAAGGAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-5b:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTTTA GTTCCGAGCT
  TGGTTCTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-5c:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTTTA GTTCCGAGCT
  TGGTTCTCGC CAAGGAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-5d:**
  5' CAGGTGTGAA CCACACCTTG GTGGAGTTTA GTTCCGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-5e:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTAAT CAAGCGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-5f:**
  5' CAGGTGTGAA CCACACCTTG GTGGAGTAAT CAAGCGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67 U18C,A344G:**
  5' CAGGTGTGAA CCACAGGAGC CACGAGTTTA GTTCCGAGGA
  ACCAACTGCG GCTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67 U18C,A5G:**
  5' CAGGTGTGAA CCACAGGAGC CACGAGTTTA GCTCCGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-GNRA:**
  5' GAACCAACTG CGGTTCCAAG GGCACTCCAC AGGAACCACG
  AGTTTAGTTC TATAGTGAGT CGTATTA 3'

- **L67ΔC13:**

5' CAGGTGTGAA CCACAGGAAC CAC_AGTTTA GTTCCGAGGA
ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67ΔA10:**
  5' CAGGTGTGAA CCACAGGAAC CACGAG_TTA GTTCCGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-6:**
  5' CAGGTGTGAA CCACAGGAAC CACGTCTTTA GTTCCGAGGA
  ACCAAGAGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-7:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTCCA GTTCCGAGGA
  ACCAACTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-8**:
  5' CAGGTGTGAA CCACAGGAAC CACGAGTCCA GTTCCGAGGA
  ACCGGCTGCG GTTCCAAGGG CTAAACACCC TATAGTGAGT CGTATTA 3'

- **L67-9:**
  5' CAGGTGTGAA CCACAGGAAC CACGAGTTTA GTTCCGAGGA
  ACCAACTGCG GTTCCTGTGG CTAAACACCC TATAGTGAGT CGTATTA 3'

Highlighted – reverse T7 promoter sequence
Red – changed sequences from wild-type
CACT– GNRA loop

5) Primers used for HDV RT-PCR experiments:

- **HDV-R1** (anneals to genomic strand):
  5' TCTGTAAAGAGGAGACTGCTG 3'

- **HDV-R2** (anneals to antigenomic strand):
  5' GAGTAAGAGTACTGAGGACTG 3'

6) Primers used in the synthesis of miniHDV (Primers mHDV1 + mHDV2 were used for generating the full-length miniHDV of antigenomic (AG) polarity. Primers mHDV3 +

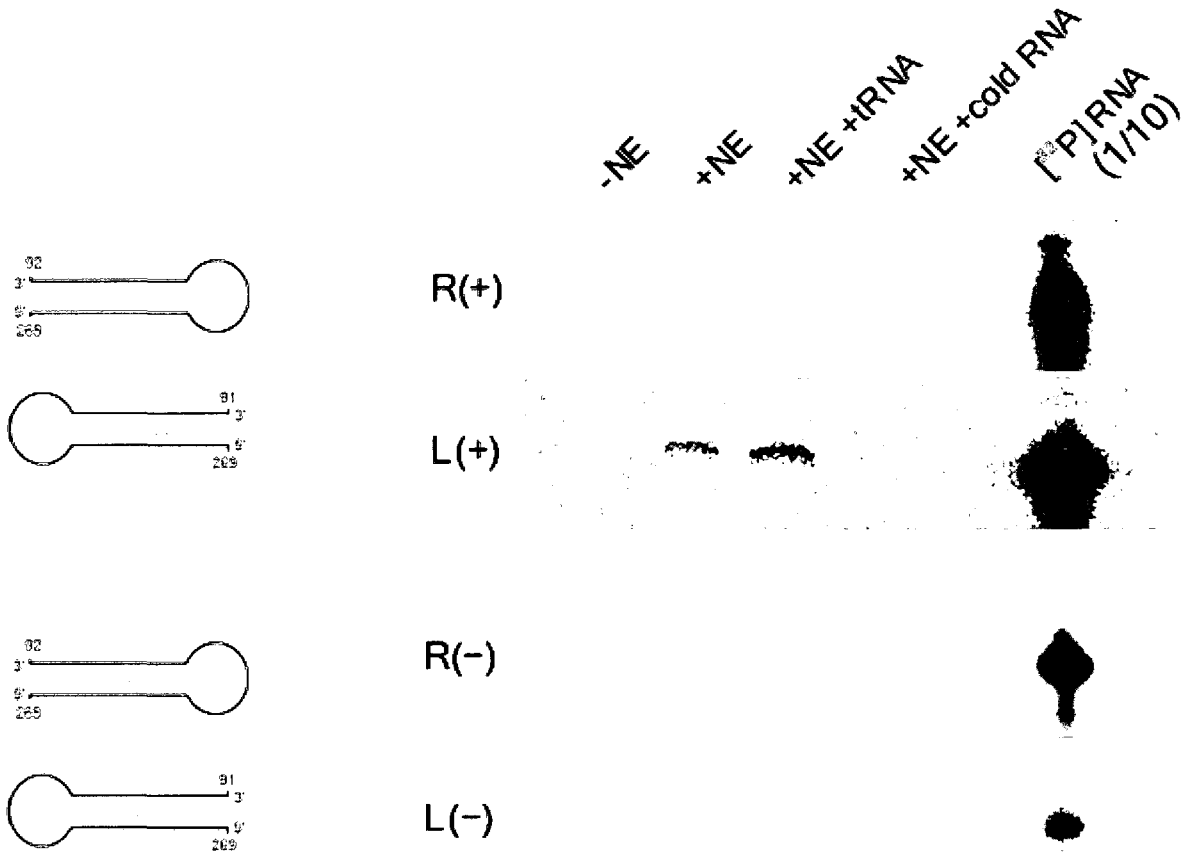
mHDV4 were used for generating the genomic (G) polarity of miniHDV. Primers mHDV2 and mHDV3 were used for first-strand AG and G synthesis, respectively, in the RT-PCR experiments.

- **mHDV1:**
  5' GGAATTCTAATACGACTCACTATAGGGCAGGTAAGAAAGGATGGAACG 3'

- **mHDV2:**
  5' TTTCTTACCTGATGGCCGGCATGG 3'

- **mHDV3:**
  5' CAGGTAAGAAAGGATGGAACG 3'

- **mHDV4:**
  5' GGAATTCTAATACGACTCACTATAGGGTTTCTTACCTGAT 3'


-NE
+NE
+NE +tRNA
+NE +cold RNA
[32P]RNA (1/10)
82
3'
5'
269
R(+)
91
3'
5'
269
L(+)
82
3'
5'
269
R(−)
91
3'
5'
269
L(−)

APPENDIX C – Supplemental figures

Fig. S.1. Interaction of RNAP II with several PSTVd-derived RNAs. Co-immunoprecipitation experiments were carried out using full-length, [γ-^{32}P]ATP end-labeled PSTVd RNA the left and right halves of both (+) and (–) polarity. Results were obtained by running immunoprecipitates on 5% polyacrylamide gel, and visualized by phosphor imaging. A 50-fold excess of tRNA was used as a non-specific competitor, and 50-fold excess of non-radiolabeled homologous RNA (cold RNA) was used as a specific competitor. One tenth of the RNAs used in each co-immunoprecipitation are used as size markers.

Curriculum Vitae

Teodora Bojić
(née Dimitrijević)

Education

- M.Sc. Biochemistry. University of Ottawa, Ottawa, ON, 2009
- B.Sc. Honours with specialization in Biochemistry (summa cum laude). University of Ottawa, Ottawa, ON, 2007
- High School Diploma. Dartmouth High School, Halifax, NS, 2002

Work experience

2007-2009	Teaching Assistant. Demonstrator for second and third year undergraduate biochemistry laboratories. Faculty of Science, University of Ottawa
2006-2007	Summer student. Laboratory of Dr. Martin Pelchat, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa
2004-2006	Part-time research assistant. Helping in computer simulations and statistical data analysis in the area of remote sensing, under the supervision of Dr. Marina Dragosevic, Terrabytes Consulting
2000-2002	Lifeguard and swimming/diving instructor. Dartmouth Sportsplex, Halifax, NS

Awards

2007-2009	NSERC – Canada Graduate Scholarship (CGS Master's)
2007-2009	University of Ottawa Excellence Scholarship
2007	Ontario Graduate Scholarship (declined)
2007	NSERC – Undergraduate Student Research award (USRA)
2006	NSERC – Undergraduate Student Research Award (USRA)
2002-2006	Dean's Honour List, University of Ottawa
2002-2006	University of Ottawa Admission Scholarship

Professional activity

Dimitrijevic, Teodora and Pelchat, Martin. "Interaction of host RNA Polymerase II with *Potato spindle tuber viroid* (PSTVd)" RiboClub Opening Session, Université de Sherbrooke, Québec. September 24, 2007. (poster presentation)

Teodora Dimitrijevic and Martin Pelchat. "Host Involvement in the replication of *Potato spindle tuber viroid*" RNA Club, University of Ottawa, October 3, 2007. (oral presentation)

Teodora Dimitrijevic and Martin Pelchat. "Interaction of host RNA polymerase II with the Potato spindle tuber viroid" Viroid 2008: International Conference on Viroids and Viroid-like RNAs in Berlin, Germany; July 26, 2008. (poster)

Dimitrijevic, Teodora and Pelchat, Martin. "Involvement of Host RNA Polymerase II in the Replication of Potato Spindle Tuber Viroid". 13th Annual Meeting of the RNA Society in Berlin, Germany; July 28 – August 3, 2008. (poster)

Teodora Dimitrijevic and Martin Pelchat. "Host Involvement in the Replication of PSTVd". RiboClub Opening Session in Sherbrooke, Canada; September 2008.

Erica Schissel, **Teodora Dimitrijevic**, Linda Rocheleau, Adam Healey, Mary Issa and Martin Pelchat. "Regulation of Iron Uptake by a Riboswitch in *Campylobacter jejuni*". RiboClub Opening Session in Sherbrooke, Canada; September 2008. (poster)

Teodora Dimitrijevic and Martin Pelchat. Evolutionary relationship between plant viroids and the human hepatitis *delta* virus. University of Ottawa RNA club in Ottawa, Canada; February 25, 2009. (oral presentation)

Skills

Laboratory skills

- Proficient in procedures including PCR, cloning, Immunoprecipitations, EMSA, HPLC, *in vitro* transcription, DNA / RNA and protein purification, radiolabeling, SDS PAGE, blotting, nuclear protein and nucleic acid extractions
- Up-to-date certification in WHMIS, laboratory, biohazard and radiation safety
- Ability to work under minimal supervision, excellent critical thinking and experimental design skills

Computer skills

- Programs in MS DOS and Windows operating systems, familiarity with UNIX
- Office packages, spreadsheats, writing and editing tools ("MS Office, "Word Perfect" Suite, "Open Office", etc.)
- Use of a variety of resources for the study of biological macromolecules, including NCBI databases, and visualization/molecular modeling tools such as "MDL Chime" and "RasMol".

- Use of Matlab

Communication skills and languages

- Excellent teamwork, leadership and communication skills
- Fluent in English, Italian and Serbo-Croatian, knowledge of German and Spanish

Other activities

- Involvement in the University of Ottawa "RNA Club"
- Member of Golden Key International Honour Society
- Italian language studies at "Scuola d'Italiano per Stranieri – Training Club" in Rome, Italy
- German language studies at "Goethe Institut" and "Cólon Fremdsprachen-Institut" in Hamburg, Germany
- Involvement in sports, Amnesty International, and high-school tutoring

References

Available upon request