

Identification and epidemiology of pospiviroids

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Identification and epidemiology of pospiviroids

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Abstract

Pospiviroids can cause serious diseases in potato and tomato. Since 1988 viroid infections have occasionally been detected in tomato crops in the Netherlands. To identify these viroid isolates unequivocally, two novel universal primer sets were designed for 'Reverse Transcription-Polymerase Chain Reaction' (RT-PCR), a sensitive technique enabling detection and identification of pospiviroids. Sequence analyses identified the viroids as *Citrus exocortis viroid* (CEVd), *Columnnea latent viroid* (CLVd) and *Potato spindle tuber viroid* (PSTVd). To find potential sources of infection, ornamental plants were screened for the presence of pospiviroids. These surveys revealed many new pospiviroid host plants as well as high infection rates for PSTVd in some ornamental species. Phylogenetic studies provided evidence that the PSTVd isolates from tomato originated from vegetatively propagated, solanaceous host plants. This conclusion was further substantiated by showing a high stability of predominant pospiviroid genotypes after mechanical pospiviroid transmission from ornamentals to potato and tomato. In addition, several experiments showed that mechanical inoculation is a likely way of pospiviroid transmission between these crops. In addition, a new pospiviroid from pepper was characterized, i.e. *Pepper chat fruit viroid* that also can infect potato and tomato. The results of above findings on pospiviroids are discussed in a broader context addressing diagnostic and epidemiological aspects as well as risk assessment in relation to quarantine measures.

Abbreviations

Viroids and viruses

ASBVd	<i>Avocado sunblotch viroid</i>	HSVd	<i>Hop stunt viroid</i>
ASSVd	<i>Apple scar skin viroid</i>	IrVd-1	<i>Iresine viroid 1</i>
CbVd-1	<i>Coleus blumei viroid 1</i>	MPVd	<i>Mexican papita viroid</i>
CCCVd	<i>Coconut cadang cadang viroid</i>	PCFVd	<i>Pepper chat fruit viroid</i>
CChMVd	<i>Chrysanthemum chorotic mottle viroid</i>	PLMVd	<i>Peach latent mosaic viroid</i>
CEVd	<i>Citrus exocortis viroid</i>	PLRV	<i>Potato leafroll virus</i>
CLVd	<i>Columnea latent viroid</i>	PSTVd	<i>Potato spindle tuber viroid</i>
CSVd	<i>Chrysanthemum stunt viroid</i>	TASVd	<i>Tomato apical stunt viroid</i>
CVd-IV	<i>Citrus viroid IV</i>	TCDVd	<i>Tomato chlorotic dwarf viroid</i>
ELVd	<i>Eggplant latent viroid</i>	TPMVd	<i>Tomato planta macho viroid</i>
GYSVd-1	<i>Grapevine yellow speckle viroid 1</i>		

General

A	adenine	ORF	open reading frame
bp	base pair	PD	pathogenic domain
C	cytosine	PEP	plastid-encoded, DNA-dependent RNA polymerase
CCR	central conserved region	Pol	polymerase
CD	central domain	PPS	Plant Protection Service
cv.	cultivar	RE	reverse
DNA	desoxyribonucleic acid	RISC	RNA-induced silencing complex
ds	double stranded	RNA	ribonucleic acid
EU	European Union	rRNA	ribosomal RNA
FW	forward	rPAGE	return-polyacrylamide gel electrophoresis
G	guanine	RT-PCR	reverse transcription-polymerase chain reaction
kbp	kilo base pair	siRNA	small interfering RNA
kDa	kilo Dalton	syn.	synonym
ME	Minimum Evolution	T	thymine
NAK	Nederlandse Algemene Keuringsdienst voor zaaizaad en pootgoed van landbouwgewassen	TCH	terminal conserved hairpin
nd	no date	TCR	terminal conserved region
NEP	nuclear-encoded, DNA-dependent RNA polymerase	TLD	terminal left domain
NPPO	National Plant Protection Organisation	TRD	terminal right domain
nt	nucleotide	U	uracil
		VD	variable domain

Chapter 1

General introduction

Discovery of viroids

In 1921 Martin (1922) described a new disease of potato (*Solanum tuberosum*) in the United States of America. The shoots of infected plants showed upright growth, branched only little and were smaller than normal. Leaves also were smaller and more pointed than those of healthy plants. Infected tubers were elongated, had more eyes, which sometimes were borne on 'knob-like protuberances' (Figure 1-1; Martin 1922). Because no causal relations to micro-organisms were found, the disease was considered of virological aetiology. The causal agent was shown to be transmitted mechanically from potato to both potato (Goss, 1926) and tomato (*Solanum lycopersicum*) (Raymer & O'Brien, 1962). At the end of the 1960s Diener & Raymer (1967; 1969) and Raymer & Diener (1969) postulated that the causal agent of potato spindle tuber disease was only a small circular RNA, based on the observed sedimentation properties of the infectivity and its sensitivity to nucleases. In addition, using electrophoretic mobility studies Diener (1971) concluded that the infectious RNA was too small to code for any proteins. Furthermore, the RNA appeared different from a satellite RNA as it replicated in host cells without a helper virus. Diener (1971) also assumed that the infectious RNA was self-replicating - despite its low molecular weight - after Montagnier (1968) had suggested the occurrence of RNA-directed RNA synthesis in virus replication. Therefore, Diener (1971) considered the infectious RNA as a primitive viral RNA that was unable to encode novel machinery in susceptible host plants for its own replication but that relied on already operative mechanisms. Because of these fundamental differences with both viruses and satellite RNAs, Diener proposed the name 'viroid' for the infectious RNA causing the spindle tuber disease. Sogo et al. (1973) visualized the potato spindle tuber viroid (PSTVd) in the electron microscope and provided further evidence for its small size. In 1978 Gross et al. succeeded in sequencing of PSTVd and predicted its secondary structure. The viroid in this case was a covalently closed circular RNA molecule of 359 nucleotides and formed a unique rod-like secondary

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TRANSMISSION, VARIATION, AND CONTROL OF CERTAIN DEGENERATION DISEASES OF IRISH POTATOES¹

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INTRODUCTION

Progress in solving the well-known problem of degeneration in the Irish potato, *Solanum tuberosum* L., has been comparatively rapid during the last decade. With this progress the apparent complexity of the problem has increased. Consequently the results of many investigators are needed and frequent reports from the various workers in this field are desirable.

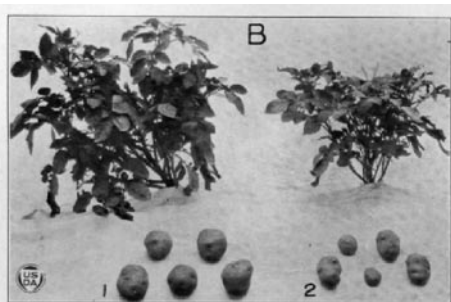


Figure 1-1. Early description of potato spindle tuber disease. Typical healthy (left) and diseased (right) plants are shown. The small, narrow shape of tubers harvested from diseased plants is clearly visible in this photo reprinted from Schultz and Folsom (1923). Courtesy of the US Department of Agriculture.

structure, in which small regions of intramolecular base pairing alternate with internal loops. As such, the final proof for the unique identity of viroids was given.

Origin of viroids

Since the first report of PSTVd as the causal agent of the potato spindle tuber disease over 40 other viroids have been reported (Table 1-1). The evolutionary origin of viroids, however, still is an enigma. Theoretically, the viroids could have been generated *de novo* in cultivated plants e.g. by mutation of cellular RNAs. However, this hypothesis is unlikely since genomic DNA of known viroid host plants does not contain viroid-related sequences (Diener 2001). Also the hypothesis that viroids are either primitive precursors or highly degenerated viral RNAs is unlikely because of the lack of nucleotide-sequence identities. The current hypothesis that viroids are relics of the pre-cellular era is more plausible. This theory is based on the recognition that RNA is the only biological macromolecule that can function both as a genotype and a phenotype, which allows its functioning in the absence of DNA and proteins (Diener, 1996). Phylogenetic analysis of viroids and the viroid-like satellite RNAs is consistent with the concept that these RNAs have a common origin, and that the viroid family *Avsunviroidae* (see below) may represent an ancestral connecting link between the two types of RNA (Elena et al., 1991). So, viroids may have originated from satellite RNAs while still being 'free-living' molecules, and have evolved in dependence on their host plants after becoming intracellular molecules (Diener, 1989).

Viroid structure, classification and nomenclature

Viroids consist of a single unit of single-stranded, covalently-closed, circular RNA, which ranges in size from 246 to 401 nucleotides depending on the viroid species (Flores et al., 2004; Tabler & Tsagris, 2004). Gross et al. (1978) reported the first sequence of a viroid (PSTVd) and predicted its secondary structure. Analysis of the sequence showed that the eukaryotic translational start codon AUG was absent, and although the prokaryotic translational start codon GUG was present, all resulting open reading frames (ORF) were way too small to encode a protein. At the lowest free energy PSTVd and most viroids form a rod-like native conformation as secondary structure, in which loops and bulges of unpaired nucleotides separate double-stranded regions. This secondary structure is assumed to be the key for biological activity by being functional as such or by providing binding signals to specific host factors (Tabler & Tsagris, 2004).

After the characterization of several viroids, Keese & Symons (1985) proposed a viroid model with a rod-like conformation consisting of five structural

Chapter 1

Table 1-1. Classification of viroids.

Avsunviroidae	
<i>Avsunviroid</i>	<i>Avocado sunblotch viroid</i>
<i>Elaviroid</i>	<i>Eggplant latent viroid</i>
<i>Pelamoviroid</i>	<i>Chrysanthemum chlorotic mottle viroid</i>
	<i>Peach latent mosaic viroid</i>
Pospiviroidae	
<i>Apscaviroid</i>	<i>Apple dimple fruit viroid</i>
	Apple fruit crinkle viroid (tentative species in the genus)
	<i>Apple scar skin viroid</i> (= dapple apple viroid; Japanese pear fruit dimple viroid; pear rusty skin viroid)
	<i>Australian grapevine viroid</i>
	<i>Citrus bent leaf viroid</i>
	<i>Citrus viroid III</i>
	Citrus viroid V (tentative species in the genus)
	Citrus viroid original source (tentative species in the genus)
	<i>Grapevine yellow speckle viroid 1</i>
	<i>Grapevine yellow speckle viroid 2</i>
	Grapevine yellow speckle viroid 3 (tentative species)
	<i>Pear blister canker viroid</i>
	Persimmon viroid (tentative species in the genus)
<i>Cocadviroid</i>	<i>Coconut cadang-cadang viroid</i>
	<i>Coconut tinangaja viroid</i>
	<i>Citrus bark cracking viroid</i> (=Citrus viroid IV)
	<i>Hop latent viroid</i>
<i>Coleuviroid</i>	<i>Coleus blumei viroid I</i>
	<i>Coleus blumei viroid II</i>
	<i>Coleus blumei viroid III</i>
	Coleus blumei viroid IV (tentative species in the genus)
	Coleus blumei viroid V (tentative species in the genus)
	Coleus blumei viroid VI (tentative species in the genus)
<i>Hostuviroid</i>	<i>Hop stunt viroid</i> (= cucumber pale fruit viroid; Citrus cachexia viroid)
<i>Pospiviroid</i>	<i>Chrysanthemum stunt viroid</i>
	<i>Citrus exocortis viroid</i> (= Indian tomato bunchy top virus)
	<i>Columnnea latent viroid</i>
	<i>Iresine viroid 1</i>
	<i>Mexican papita viroid</i>
	Pepper chat fruit viroid (tentative species in the genus)
	<i>Potato spindle tuber viroid</i>
	<i>Tomato apical stunt viroid</i>
	<i>Tomato chlorotic dwarf viroid</i>
	<i>Tomato planta macho viroid</i>

Unassigned viroids: blueberry mosaic viroid-like RNA, burdock stunt viroid, *Nicotiana glutinosa* stunt viroid, pigeon pea mosaic mottle viroid and tomato bunchy top viroid (a name also used as a synonym for *Potato spindle tuber viroid*).

domains on the basis of nucleotide sequence analysis. These domains included a central (CD), pathogenic (PD), variable (VD), terminal left (TLD) and terminal right (TRD) domain (Figure 1-2A). Initially, the domains proposed by Keese & Symons were presumed to have functional roles. However, the situation appeared more complex since Sano et al. (1992) concluded that pathogenicity was not only controlled by the PD but also by determinants located within TLD, VD, and TRD. Later it was shown that even substitutions of single nucleotides in the lower CD could have a dramatic impact on pathogenicity (Qi & Ding, 2003a; Wassenegger et al., 1996). The model of structural domains of Keese & Symons still holds for the majority of viroid species that are currently characterized, in particular those classified in the family of the *Pospiviroidae*. Only a few viroids are more branched, and are assigned to the family of the *Avsunviroidae* (Figure 1-2B). Two of these, *Avocado sunblotch viroid* (ASBVd) and *Eggplant latent viroid* (ELVd) adopt quasi-rod-like structures, whereas *Peach latent mosaic viroid* (PLMVd) and *Chrysanthemum chlorotic mottle viroid* (CChMVd) fold into clearly branched secondary structures (Flores et al., 2004).

For the members of the family *Pospiviroidae* the core of the CD contains two highly conserved fragments, one in the upper and one in the lower strand, which together form the Central Conserved Region (CCR) (Flores et al., 1997). In total, five different CCR types are known. In addition to the CCR, each member of the family *Pospiviroidae* also contains one of two highly conserved sequence fragments in the TLD, the so-called Terminal Conserved Hairpin (TCH) and the Terminal Conserved Region (TCR). The presence of either a TCH or a TCR and the type of CCR form the main genus demarcation criteria within the *Pospiviroidae* (Flores et al., 2005b). These criteria essentially lead to the same grouping as would be obtained after phylogenetic analyses of nucleotide sequences from complete viroid genomes. Members of the family *Avsunviroidae* lack these conserved regions. In stead, they are able to form hammerhead structures, consisting of 11 conserved residues and adjacent helices (Figure 1-2B) that mediate the self-cleavage of their multimeric-RNA intermediates of both polarities (Flores et al., 2000a).

In addition to the rod-like structure, members of the family *Pospiviroidae* can adopt metastable secondary structures containing hairpins (Riesner, 1991). Hairpin I constitutes of the core nucleotides of the CCR upper strand and the flanking inverted repeat, and includes a terminal tetraloop, the adjacent 3 bp-stem, a small loop and a long stem of 9-12bp (Figure 1-2A). Hairpin II is formed by sequences at the lower strand of the rod-like structure at both sides of the CCR. It comprises a stem of 11-12 bp and a large loop varying from 82 to 168nt. Finally, many members of the *Pospiviroidae* contain an internal loop in the CCR (for PSTVd nt 98-102 and 256-261) that is highly homologous to loop E of the eukaryotic 5 S rRNA (Branch et al., 1985). Because this E-loop is involved in the synthesis and transport of 5 S rRNA, a similar role is assumed for the E-loop-like structure of the *Pospiviroidae* (Baumstark et al., 1997; Schrader et al., 2003).

Viroid replication

The different genome structures of members of the *Pospiviroidae* and *Avsunviroidae* correlate with differences in their replication process, i.e. both the subcellular localization, the pathway of replication, the enzymes - including the capacity for self-cleavage mediated by ribozymes - and the RNA motifs involved in replication (Table 1-2).

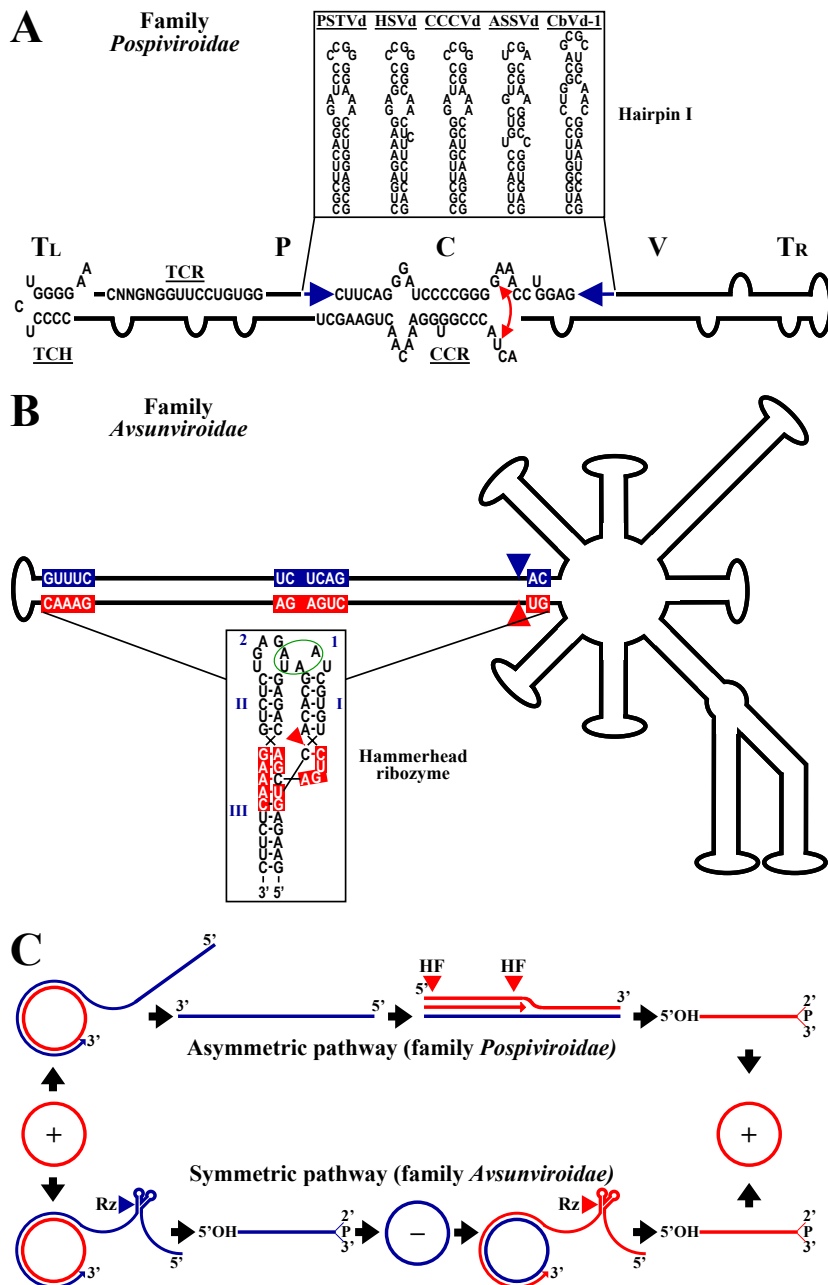
First, the replication of the *Pospiviroidae* and *Avsunviroidae* takes place in different cell organelles. Members of the *Pospiviroidae* replicate in the nucleus as has been shown by subcellular fractionation studies and in situ hybridization experiments (e.g. Spiesmacher et al., 1983; Harders et al., 1989; Qi & Ding, 2003b). In contrast, members of the *Avsunviroidae* replicate in chloroplasts (e.g. Bonfiglioli et al., 1994; Lima et al., 1994; Navarro et al., 1999).

Secondly, the principle of replication differs (Figure 1-2C). Although members of both families replicate via a rolling circle mechanism, the *Pospiviroidae* follow an asymmetric pathway, whereas the *Avsunviroidae* replicate via a symmetric

Table 1-2. Main characteristics of members of the families *Pospiviroidae* and *Avsunviroidae*.

Features	<i>Pospiviroidae</i>	<i>Avsunviroidae</i>
Secondary structure	Rod-like	Quasi rod-like or branched
Conserved sequence regions	Present (CCR, and TCH or TCR)	Absent
Replication site	Nucleus	Chloroplast
Replication pathway	Asymmetric cyclus	Symmetric cyclus
Replication enzymes	DNA dependent RNA polymerase II	Nuclear-encoded (chloroplastic) RNA polymerase and/or chloroplastic encoded RNA polymerase
Cleavage	No self-cleavage domain	Self-cleavage via hammerhead structures
Number of host plant species	Moderate	Low

Figure 1-2. Genome structure and replication mechanism of viroids (Darós et al., 2006; slightly modified). (A) Scheme of the rod-like genomic RNA that is characteristic of the family *Pospiviroidae* with the central (C), pathogenic (P), variable (V), and terminal left and right (TL and TR, respectively) domains. The central conserved region (CCR; genus *Pospiviroid*), the terminal conserved region (TCR; genera *Pospiviroid*, *Apescaviroid* and part of *Coleviroid*) and the terminal conserved hairpin (TCH; genera *Hostuviroid* and *Cocadviroid*) are shown. Blue arrows indicate the flanking sequences that, together with the upper strand of the CCR, form hairpin I, as depicted in the inset box for the type species of the five genera of the family *Pospiviroidae*: *Potato spindle tuber viroid* (PSTVd), *Hop stunt viroid* (HSVd), *Coconut cadang-cadang viroid* (CCCVd), *Apple scar skin viroid* (ASSVd) and *Coleus blumei viroid* 1 (CbVd-1). The red double-headed arrow connects two residues of PSTVd linked after ultraviolet irradiation as a consequence of forming part of loop E. (B) Scheme of the branched genomic RNA of *Peach latent mosaic viroid* (PLMVd; family *Avsunviroidae*), in which the sequences conserved in most natural hammerhead ribozymes are shown on a red and blue background for (+) and (–) polarities, respectively, and the self-cleavage sites are indicated by arrowheads. The structure of the (+) hammerhead ribozyme is shown in the inset box, with Roman and Arabic numerals depicting helices I, II and III, and loops 1 and 2, respectively, and the arrowhead indicating the self-cleavage site. The green oval indicates a tertiary interaction between loops 1 and 2 that enhances catalytic activity. (C) Asymmetric and symmetric pathways of the rolling-circle replication mechanism that is used by members of the families *Pospiviroidae* and *Avsunviroidae*, respectively. Red and blue lines refer to (+) and (–) strands, respectively. Arrowheads point to cleavage sites of a host factor (HF) or ribozymes (Rz), and the resulting 5' and 3' groups are indicated.



rolling circle mechanism (Branch & Robertson, 1984; Daròs et al., 1994; Navarro et al., 1999). For the *Pospiviroidae*, the (+)-circular RNA is iteratively transcribed into oligomeric, linear (-)-strand RNAs. These strands then serve as intermediates for the synthesis of oligomeric, linear (+)-strand RNAs, which are cleaved into unit-length monomers (see below) and then ligated into circles. For the *Avsunviroidae*, the circular (+)-RNA is transcribed into linear, oligomeric (-)-strand RNAs, which are cleaved into unit-length molecules that circularize. The circular (-)-RNA then serves as a template for the synthesis of linear, oligomeric (+)-strand RNAs, which subsequently are cleaved into unit-length, circular monomers.

Thirdly, different enzymes (and ribozymes) are involved in replication, i.e. transcription, cleavage and ligation of the RNA. Nuclear DNA-dependent RNA polymerase II (Pol II) is essential for transcription of PSTVd and other members of the family *Pospiviroidae*. Mühlbach & Sanger (1979) showed that α -amanitin, a fungal toxin that in the nanomolar range inhibits Pol II, inhibits the replication of PSTVd in tomato protoplasts. In addition, Rackwitz et al., (1981) showed that purified Pol II from tomato could transcribe the (+)-PSTVd RNA template *in vitro*, whereas Schindler & Mühlbach (1992) excluded a role for both Pol I and III. The transcription initiation site in the circular (+)-RNA template of PSTVd was recently mapped to U359 or C1 (Kolonko et al., 2006). The initiation site at the (-)-strand template, however, still remains to be determined. In addition to the replication-initiation site in the left terminal domain, two GC boxes may play a role in the transcription of PSTVd (Fels et al., 2001). For *Avsunviroidae* two polymerases have been related to the transcription in chloroplasts. The nuclear-encoded, DNA-dependent RNA polymerase (NEP) has been shown to be involved in the transcription of ASBVd (Navarro et al., 2000) and PLMVd (Delgado et al., 2005; Rodio et al., 2007), whereas the plastid-encoded DNA-dependent RNA polymerase (PEP) has been related to the transcription of PLMVd (Pelchat et al., 2002; Motard et al., 2008). The transcription initiation sites for ASBVd were mapped to U121 and U119 on the (+)- and (-)-RNA, respectively, and for PLMVd between A50 and C51 and to U284 on the (+)- and (-)-RNA, respectively. The first are situated at terminal loops while the latter are situated at a conserved region of the viroid molecule in the vicinity of the PLMVd self-cleavage sites (Navarro & Flores, 2000; Delgado et al., 2005; Motard et al., 2008). With regard to cleavage and ligation also different enzymes are involved. For *Pospiviroidae*, cleavage is supposed to be catalyzed by a yet unknown host enzyme. During viroid processing the first cleavage of PSTVd might occur within the stem of a GNRA tetraloop between the nucleotide positions G95 and G96. Subsequently, a conformational change switches the tetraloop motif into a loop E motif, stabilizing a base-paired 5' end. The second cleavage yields unit-length linear intermediates, whose 3' end is also base-paired (Baumstark & Riesner, 1995; Baumstark et al., 1997). However, such a mechanism of cleavage and ligation probably does not apply to members that cannot form the GNRA tetraloop, such as *Apple scar skin viroid* (ASSVd). Therefore, Gas et al. (2007)

concluded that the hairpin I/double-stranded structure formed by the upper CCR strand and its flanking nucleotides are involved in cleavage of different members of the *Pospiviroidae*. After a second conformational switch, ligation takes place by a yet unknown host enzyme (Figure 1-3). These data confirm the model proposed by Diener (1986) of a thermodynamically, extremely stable based-paired conformation with a prominent role for the CCR and hairpin I in promoting the adoption of this structure. In contrast, the members of the *Avsunviroidae* do not need host enzymes for cleavage because they can form hammerhead structures that function as ribozymes, i.e. self-cleaving RNAase activity. These structures are located in both (+) and (-) RNA strands and autocatalyse self-cleavage *in vitro* and *in vivo* (Flores et al., 2000a; Hernández & Flores 1992; Hutchins et al., 1986; Lafontaine et al., 1995). Concerning ligation, it is still unclear whether the circularization of monomers is an autocatalytic process or needs a chloroplastid RNA ligase (Flores et al., 2005a).

Finally, different conserved RNA motifs are involved in the replication of the *Pospiviroidae* and *Avsunviroidae*. For members of the *Pospiviroidae*, the sequence and structural conservation of the CCR is essential for processing of the viroid (Baumstark et al., 1997; Gas et al., 2007). In addition, Zhong et al. (2008) demonstrated the role of the TLD in replication by enlarging or deleting the first four loops. For members of the *Avsunviroidae*, the small conserved motifs that can form hammerhead structures are essential for viroid replication (Flores et al., 2000a). In conclusion, both the genome structure and catalytic activities of the viroid RNA and their interaction with host-cellular components determine the course of the replication process.

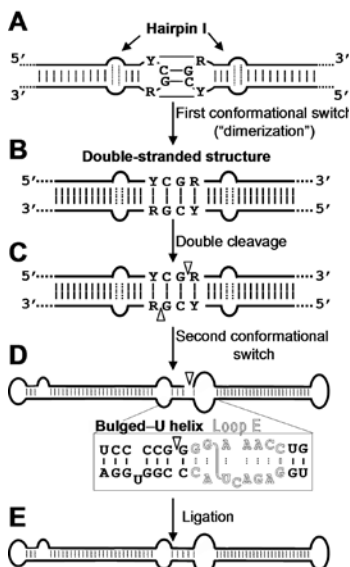


Figure 1-3. Model for processing *in vivo* of the oligomeric (+) replicative intermediates of the family *Pospiviroidae* (Gas et al., 2007). The model envisages a kissing loop interaction between the palindromic tetraloops of two consecutive hairpin I motifs (A), with their stems forming subsequently a longer interstrand duplex (B). This double-stranded structure is the substrate for cleavage at specific positions in both strands (C). Following a second conformational switch the resulting unit-length strands adopt the extended rod-like structure with loop E (in outlined fonts) and the adjacent bulged-U helix (D), which is the substrate for ligation (E). R and Y refer to purines and pyrimidines, respectively, the S-shaped line denotes the UV-induced cross-link, and white arrowheads mark the cleavage sites in the double-stranded structure and the ligation site in the extended conformation.

Viroid movement

Following replication, viroid RNAs have to spread within the plant to establish infection. The process of spreading starts with intracellular transport from the site of genesis (nucleus, chloroplast) and is followed by cell-to-cell and long-distance movement.

The intracellular movement of viroids from and to the sites of replication is still poorly understood. The viroid RNAs may have a – still unidentified – signal that directs the entrance into the nucleus (*Pospiviroidae*) or chloroplast (*Avsunviroidae*), for example by changing the permeability of the membranes. Alternatively, the viroid RNAs may possess motifs that enable entry and exit of the respective organelles (Ding & Itaya, 2007). In addition, certain nuclear-encoded chloroplastic proteins have been presumed to facilitate transport of ASBVd into the chloroplast (Daròs & Flores, 2002; Tsagris et al., 2008).

Similarly to plant viruses, trafficking of viroids from initially infected cells to neighbouring cells occurs via plasmodesmata, whereas long-distance trafficking into selective sink organs uses the phloem (Palukaitis, 1978; Ding et al., 1997; Zhu et al., 2001). The principle of long-distance trafficking, however, is different because viroids lack movement proteins like those used by plant viruses. As a consequence, viroids depend on interactions with host cellular components for long-distance trafficking. So far, four cellular components have been proposed. Lectin PP2 from phloem cells of cucumber (*Cucumis sativus*) has been shown to bind *Hop stunt viroid* (HSVd) both *in vitro* and *in vivo*. As a result, the complex moved from rootstocks into scions suggesting that PP2 from cucumber contributes to long-distance trafficking of HSVd RNAs (Gómez & Pallás, 2001 and 2004; Owens et al., 2001). In addition, VirP1, a protein from tomato and many other solanaceous plant species, was shown to interact *in vitro* with the so-called RY motifs in the right terminal domain of PSTVd and HSVd (Maniataki et al., 2003). These RY motifs also occur in other members of the genus *Pospiviroid* and a few more members of other genera within the *Pospiviroidae* (Gozmanova et al., 2003). Mutations in these RY motifs resulted in loss of both VirP1 binding and viroid infectivity (Gozmanova et al., 2003; Hammond, 1994). Finally, Gómez et al. (2005) identified two proteins from melon, i.e. CmmLec17 and an unidentified 14-kDa protein that were able to bind ASBVd. However, evidence on the role of these proteins in long-distance viroid trafficking still has to be provided.

From the viroid side, also the metastable hairpin II of members of the genus *Pospiviroid* seems to be involved in trafficking. Initially, hairpin II was presumed to be involved in replication because mutations inhibiting the formation of this hairpin were lethal or reverted to wild-type viroid in mechanically inoculated plants (Loss et al., 1991; Owens et al., 1991). However, several mutations predicted to disrupt the hairpin II core region did not prevent PSTVd replication in *Nicotiana benthamiana*

protoplasts (Zhong et al., 2008), thus indicating interference of hairpin II with viroid trafficking.

Based on the available information, it has to be concluded that knowledge on movement of viroids in plants is still fragmentary.

Symptom expression

The pathogenicity of viroids has been assumed to result from interactions between their genomic RNAs, or derivatives thereof, and host component(s). These interactions do not stand alone, since they are influenced by external conditions, especially temperature. Besides this conventional theory, a role for RNA silencing in viroid symptom development has been proposed recently.

Viroid RNA structural elements

The role of the viroid structure in symptom development has been demonstrated by Schnolzer et al. (1985), who showed that four nucleotide substitutions in the PD of PSTVd converted an 'intermediate' strain into a 'severe' strain. In addition, Owens et al. (1996) revealed a correlation between the secondary and tertiary structure of part of the PD of PSTVd and pathogenicity. Also for *Citrus exocortis viroid* (CEVd) a relation between the nucleotide sequence of the PD and symptom development was shown (Visvader & Symons, 1985 and 1986). However, pathogenicity determinants for *Pospiviroidae* are not restricted to the PD. After constructing interspecies chimeras between CEVd and *Tomato apical stunt viroid* (TASVd), Sano et al. (1992) concluded that pathogenicity was controlled by determinants located within the TLD, PD, VD, and TRD. In addition, Rodriguez & Randles (1993) showed that pathogenicity of *Coconut cadang cadang viroid* (CCCVd) was related to sequence mutations in both the PD and the lower strand of the CD. Later Wassenegger et al. (1996) and Qi & Ding (2003a) showed that even substitutions of single nucleotides in the lower strand of the CD, i.e. C259U and U257A, had a dramatic impact on pathogenicity, including both symptomatology and host range. Although some specific nucleotide positions have been related to pathogenicity, this still cannot fully explain the variation in symptomatology.

Also for *Avsunviroidae* still little is known on pathogenicity determinants. Conversion of the tetraloop 82UUUC85 to 82GAAA85 in the branched conformation of CChMVd changed infections with severe symptoms to symptomless infections (De la Peña & Flores, 2002). In addition, variants of PLMVd with an insertion of 12-13 nucleotides forming a hairpin in loop A between positions 337 and 1 induced peach calico, an extreme chlorosis, whereas this hairpin is absent in PLMVd isolates not causing this symptom (Malfitano et al., 2003). However, also for the *Avsunviroidae* an overall theory relating viroid structure and symptomatology is lacking.

Host components

Host components, especially nucleotide sequences complementary to viroid sequences have also been assumed to contribute to the symptom development. Alignments of sequences from viroids and cellular RNAs revealed identities with two functional RNAs. The highest identities were found in the lower strand of the CD of PSTVd and the 5' terminus of a small nuclear RNA homologous to mammalian U1 RNA (Diener, 1981), and in the neighbouring parts of the lower strands of the PD and CD of PSTVd and the 7S RNA from tomato (Haas et al., 1988). Complex formation between the PSTVd minus strand and U1 or 7S RNA was supposed to interfere with pre-rRNA processing or with the formation of signal recognition particles, respectively (Diener, 2001). These results, however, do not explain the difference in symptom expression in tomato plants between mild and severe strains of the same viroid species that reach similar titers. There are indications, however, that a plant-encoded 68 kD protein kinase might direct symptom induction. First Hiddinga et al. (1988) showed that PSTVd activated this enzyme, and later Diener et al. (1993) demonstrated *in vitro* that the mammalian protein kinase P68 was strongly activated by viroids strains that incite moderate to severe symptoms, but far less by a mild strain. Being the plant counterpart of P68, Diener (1993) expected a similar effect for the host-encoded 68 kDa protein.

RNA-silencing

As an alternative for understanding viroid symptom development, RNA silencing is considered to be involved in symptom expression. Papaefthimiou et al. (2001) showed that PSTVd replication is accompanied by the synthesis of small interfering RNAs (siRNAs), derived from both the plus and minus strands, which are presumably incorporated into the RNA-induced silencing complex (RISC). PSTVd itself seems to escape siRNA-mediated degradation because high levels of siRNAs were associated with accumulation of viroid genomic RNAs. This might be explained by their highly ordered secondary structure, which is supposed to be resistant to RISC-mediated cleavage (Itaya et al., 2007, Wang et al., 2004). However, the results of Carbonell et al. (2008) indicating that mechanical co-inoculation of the viroid RNA with an excess of homologous dsRNA induced significant reduction of the infectivity for CEVd, PSTVd and CChMVd, suggests the involvement of RISC. In addition, RISC appears to be mainly active in the cytoplasm, where the siRNAs from PSTVd-infected plants are also found. The latter do not occur in the nucleus, the location of pospiviroid replication (Denti et al., 2004). As a consequence, RISC may not target PSTVd at its place of replication and accumulation. On the other hand Wang et al. (2004) demonstrated that symptom development is correlated with the production of siRNAs and assumed that these siRNAs mediate silencing the expression of some physiologically important host genes resulting in the occurrence of symptoms. In line with this hypothesis, high levels of siRNAs increased symptom severity for

PSTVd and ASBVd (Itaya et al., 2001; Markarian et al., 2004). Moreover, Wang et al. (2004) also found a correlation between symptom development and the production of siRNAs in transgenic tomato plants expressing non-replicating dsPSTVd RNAs. Schwind et al. (2009), however, using the progeny of the same transgenic tomato plants used by Wang et al. (2004), did not observe symptoms in non-inoculated plants despite the accumulation of high levels of siRNA. In conclusion, a role for RNA-silencing in symptom development has been postulated, but was not confirmed in subsequent experiments.

Summarizing the information on the role of viroid RNA structural elements, host components and RNA-silencing, it must be concluded that no conclusive theory has been provided so far, which explains the principle of symptom expression in viroid infection.

Transmission and epidemiology

Viroids can be transmitted in four different ways. Vegetative propagation of infected plants is the most efficient way since all new plants are derived from an infected source via bulbs, cuttings, grafts, micro-plants, rhizomes and tubers. Once established, the infection persists and therefore, vegetatively propagated plants from infected lots act as permanent sources of inoculum. Moreover, the absence of symptoms, often the case in ornamentals (Verhoeven et al., 2008a, b and c), increases the risk of using infected plants for propagation. Vegetative propagation has been the major way of PSTVd transmission in potato and ornamentals such as *Brugmansia* spp. and *Solanum jasminoides* (Owens et al., 2009; Verhoeven et al., 2010).

Mechanical transmission is the second important way of viroid spread (e.g. Hadidi et al., 1997; Hollings & Stone, 1973; Manzer & Merriam, 1961; Van Dorst & Peters, 1974). Under favourable conditions, pospiviroids may be readily transmitted through the usual crop handling. This is most clearly seen in tomato crops where viroids mainly spread along the row in the direction of crop handling (Verhoeven et al., 2004).

A third way of viroid transmission is via infected seeds and pollen. For several viroids transmission by seed has been reported (Chung & Pak, 2008; Fernow et al., 1969; Haddidi et al., 1991; Kryczynski et al., 1988; Singh, 1970; Singh & Dilworth, 2009; Singh et al., 1992b). PSTVd is assumed to have spread via infected true seed among potato germplasm collections all over the world. Once present in a germ bank, the viroid could be transmitted to other (wild) potato plants either mechanically or by pollen exchange. Furthermore, seeds may be an important source of infection for crops mainly propagated generatively such as pepper and tomato.

Finally, insects may contribute to the transmission of viroids. Aphid transmission has been reported for PSTVd by De Bokx & Piron (1981) and for *Tomato planta macho viroid*, TPMVd (Galindo et al., 1986). However, these data need confirmation. For PSTVd transmission by aphids has been confirmed in the case of mixed infection by *Potato leafroll virus* (PLRV; Querci et al., 1997; Syller et al., 1997). It is assumed that viroid RNAs become transcapsidated by the coat protein of PLRV, thereby enabling the viroid to escape digestion in the gut of the aphid (Querci et al., 1997). Besides aphids, only bumble bees have been reported to transmit TASVd and TCDVd (Antignus et al, 2007; Matsuura et al., 2010). It is not clear, however, whether transmission results from mechanical transmission by wounding of flowers or from pollination.

The epidemiology of viroids depends on several factors like the extent of the host plant range, the number of infected plants and the potential ways of transmission. Within a plant species all ways of transmission may be applicable, whereas between species only mechanical and insect transmission may apply. Within vegetatively propagated crops such as potato and many ornamental host plants, infected seed potatoes and cuttings mainly account for transmission, respectively. In addition, mechanical transmission and insects may account for introductions and further spread of viroids in a crop. In crops grown from true seed such as pepper and tomato, viroids have to be introduced in each new cultivation via infected seed, mechanical transmission or insects. The number of known potential sources of pospiviroid infection other than infected seeds has increased dramatically since many new viroid host plant species have been identified and high numbers of infected plants per species have been reported during the last few years (Table 7-1; Verhoeven et al., 2008a, b, c, and 2010). Since many sources of viroid inoculum exist, more new outbreaks can be expected in the near future.

Economic impact of viroids

Although there are still many unanswered questions on the background of viroid symptomatology and epidemiology, there are no doubts about the economic impact of viroids. The reason for their discovery was the fact that they caused serious diseases in main fruit, vegetable and ornamental crops. Affected plants show various symptoms like growth reduction, discoloration, distortion and necrosis (Figure 1-4A/E) that result in direct financial losses in commercial production systems. In addition, also indirect losses may be considerable.

Direct losses

Direct losses result from both yield and quality reduction and have been reported for many viroids. In the Philippines CCCVd killed many coconut palms



Figure 1-4. Viroid infected crops. (A) Coconut trees in the Philippines showing various degree of decay, a disease caused by *Coconut cadang-cadang viroid*, courtesy of JW Randles. (B) *Hop stunt viroid* causes growth reduction of hop as shown by the plants in the centre compared to the vigorous plant in the front, courtesy of T Sano. (C) Secondary infections of *Potato spindle tuber viroid* on tubers grown from infected seed potatoes: tubers are elongated, severely malformed and show some protuberances. (D) Chlorosis (left), which proceeds to necrosis (right) in tops of a tomato plants infected by *Tomato chlorotic dwarf viroid*. Similar symptoms may be caused by other pospiviroids infecting tomato, including *Potato spindle tuber viroid*. (E) Premature flowering and growth reduction of the group chrysanthemum plants infected by *Chrysanthemum stunt viroid*, left in the front. (F) lot of *Lycianthes rantonnetii*, symptomlessly infected by *Potato spindle tuber viroid*.

(Figure 1-4A). Since infected trees ceased nut production at an average of five years before they eventually died, and newly planted trees took another five to eight years to start producing nuts, losses concerned a period of 10 to 13 years. Calculations revealed that since 1950 losses due to CCCVd infections mounted up to 66 million USD per year (Randles & Rodriguez, 2003). In Japan HSVd (Figure 1-4B) reduced the numbers of hop cones by 30-50%, whereas their mean weight was reduced to 66% (Sano, 2003). In the last two decades of the 20th century in Russia the yield and the quality (Figure 1-4C) of seed potatoes decreased dramatically because of PSTVd infections up to 70% (Kastalyeva et al., 1992; Owens et al., 2009). In North America yield losses by PSTVd infections in potato have been calculated to be approximately 1% over the last century. This seems low but nevertheless accounted for considerable losses regarding the large scale of potato production (Owens & Verhoeven, 2009). Under experimental conditions yield reductions from 17 to 64% have been reported for PSTVd in potato; lower yield reductions being caused by mild strains and higher reductions by severe strains (Kowalska-Noordam et al., 1987; Pfannenstiel & Slack, 1980; Singh et al., 1971). In tomato the yield reduction by PSTVd (Figure 1-4D) of five experimentally inoculated varieties varied from 0 to 57% (Kryczynski et al., 1995). In chrysanthemum (*Chrysanthemum x morifolium*) *Chrysanthemum stunt viroid* (CSVd) has been reported to reduce plant size (Figure 1-4E) up to 65% (Horst et al., 1977).

Also external conditions contribute to the yield reduction, e.g. temperature and nutrition. For example, Sano (2003) reported that stunting of hop infected by HSVd in the warmer south of Japan was more severe than in the cooler north. Skoric et al. (2001) and Handley & Horst (1988) reported similar effects of temperature on the symptomatology of CEVd and CSVd, respectively. Concerning plant nutrition, nitrogen has been found to increase the severity of CEVd symptoms (Weathers, 1964), and a similar effect has been reported for manganese and PSTVd (Lee & Singh, 1972).

Viroids also may reduce the quality of the products of the infected crops. HSVd reduced the alpha acid content in hop cones by 50% (Sano, 2003), thus reducing the quality for beer production. ASSVd caused scar skin or dapple symptoms on apples and rusty skin on pears, all degrading the fruit quality (Chen et al., 1987; Liu et al., 1957). In the ornamental industry CSVd degrades the quality of chrysanthemum cut flowers by causing discoloration and malformation (Brierley & Smith, 1951).

The total sum of the direct losses results from multiplication of the number of affected plants and the yield and/or quality reduction per plant. In addition, they are also related to the level of knowledge on the viroid. In the case of a new disease, little will be known on the symptomatology, cause and control. Growers often will not recognize or misinterpret symptoms and will take inadequate control measures. For example, when PSTVd was detected in tomato in New Zealand for the first time (Elliott et al., 2001; Verhoeven et al., 2004), it took more than one year to identify

the cause of the disease. During this period no effective control measures had been taken and as a consequence, the disease had spread all over the greenhouse. In contrast, an outbreak of PSTVd in tomato in Belgium was restricted to 20 plants in two rows, by eradicating these and a few adjacent rows at an early stage (Verhoeven et al., 2007a).

Indirect losses

In addition to losses directly related to yield and quality reduction, indirect effects may have a serious economic impact. Indirect losses include extra costs for measures to prevent and control viroid diseases. Prevention of the introduction of a viroid in a field, greenhouse, area or country is the best safeguard for a healthy crop. The first step in prevention includes the production of viroid-free planting material, i.e. both seeds and plants for planting. This requires measures on hygiene and testing to safeguard the viroid-free status at considerable costs. In addition, the costs to acquire and disseminate knowledge on viroids contribute to the indirect losses. Both industries and governments have to cope with such indirect losses to prevent direct losses and safeguard national and international trade, respectively.

For non-indigenous viroids or those with only a restricted spread, governments may raise trade barriers (quarantine status) to prevent the introduction, establishment or further spread of these viroids. In the European Union (EU) three viroids are regulated at this moment (EU Council Directive 2000/29/EC, n.d.). For CCCVd and PSTVd, the introduction and spread should be prevented in all host plants; for CSVd, which already occurs in the EU, only the spread in chrysanthemum has to be prevented. By enacting these regulations governments aim to prevent large direct losses by serious, non-indigenous or contained plant pests and diseases. However, such regulations often cause extra barriers and costs for trading. For example, both seed potatoes and true potato seeds may only be imported into the EU when subjected to post-entry quarantine inspection and testing for regulated 'organisms', including PSTVd. In 2006 and 2007, in the Netherlands also ornamental plants infected by PSTVd had to be traced (governmental costs) and destroyed (costs for the industry). Although these plants did not suffer any noticeable losses themselves, these measures had to be taken because these ornamentals might act as sources of infection for potato and tomato (Figure 1-4F). Total costs for the ornamental industry were estimated between 3 and 5 million Euros, including destroying of plants, cleaning, disinfection and purchase of new plants. The costs for the government for inspection, testing and eradication measures were estimated € 700,000.-- in one year. Since then, plant passport requirements apply for these ornamental crops, which amounts for the Dutch ornamental industry to circa € 50,000.-- per year (De Hoop, et al., 2008).

Scope of investigation

Since 1988 viroid infections have occasionally been detected in tomato crops in the Netherlands. The presence of the viroids in these symptomatic tomatoes was established by return-polyacrylamide gel electrophoresis (r-PAGE, Huttinga et al., 1987). The identity of the viroids, however, could not be determined with this method. Only the first isolate, found in 1988, was additionally sequenced and identified as PSTVd-N, a genotype clearly deviating from previously sequenced PSTVd genotypes (Puchta et al., 1990). In 1995 PSTVd-N and eight other viroid isolates from tomato were tested by Agdia (Elkart, IN - USA) by hybridisation with DIG-labelled RNA probes for CEVd, CSVd, PSTVd, TASVd and TPMVd. Besides isolate N, only one other isolate reacted with the PSTVd-specific probe, four isolates hybridised with a probe for CEVd and the other isolates did not react with any of the probes. This raised the question whether these isolates belong to another of the known viroid species or represent one or more new viroid species. Moreover, tracing the origins of infection neither revealed any relation with seed lots nor with plants for planting.

So, the main questions addressed in this thesis are: (i) is it possible to develop a sensitive and unequivocal diagnostic method for pospiviroids, (ii) could the identifications be used to derive the origin or source of pospiviroid infections in tomato, and (iii) what is a likely route of transmission of pospiviroids between plant species?

To identify the viroids isolated from tomato, two universal pospiviroid primer sets were designed for 'Reverse Transcription-Polymerase Chain Reaction' (RT-PCR). Unc cloned PCR products were sequenced, and sequence analyses enabled identification of all viroids in Dutch tomato crops as CEVd, *Columnnea latent viroid* (CLVd) or PSTVd (Chapter 2). Since CLVd only had been identified in three ornamental species, RT-PCRs with the newly developed primer sets were used in surveys to screen ornamental plants for the presence of pospiviroids (Verhoeven et al., 2008a). These and subsequent surveys for PSTVd revealed many new pospiviroid host plants as well as high infection rates for PSTVd in some ornamental species (Chapter 3 and 7). Furthermore, phylogenetic studies provided evidence that the PSTVd isolates from tomato originated from vegetatively propagated, solanaceous host plants. This conclusion was further substantiated when high stability of predominant pospiviroid genotypes was shown after mechanical pospiviroid transmission from ornamentals to potato and tomato (Chapter 4). In addition, mechanical transmission was studied to reveal its possible role in transfer of pospiviroids, in particular PSTVd, between these species (Chapter 5). Furthermore, a new pospiviroid from pepper was characterized by describing its biological and molecular features (Chapter 6). In Chapter 7 the results from the experimental chapters are discussed in a broader context addressing diagnostic and epidemiological aspects as well as risk assessment in relation to quarantine.

Chapter 2

Natural infections in tomato by *Citrus exocortis viroid*, *Columnea latent viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*

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Abstract

Since 1988 viroids have been occasionally detected in samples of tomato (*Solanum lycopersicum*) originating both in the Netherlands and other countries. Infected plants showed chlorosis, bronzing, leaf distortion and growth reduction. Initial diagnosis of these viroids was by return-polyacrylamide gel electrophoresis, which did not allow a further identification. This paper reports upon the identification of these viroids by reverse transcription-polymerase chain reaction and sequence analysis. Three known viroids of tomato, i.e. *Citrus exocortis viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid* were identified. In addition, six isolates were identified as *Columnnea latent viroid*, a viroid so far only detected in some ornamental species. Like the isolates previously isolated from ornamental species, the isolates from tomato share genetic characteristics of both the genera *Hostuviroid* and *Pospiviroid*. The biological characteristics of all four viroids, especially their potential effects on both potato (*Solanum tuberosum*) and tomato, stress the need for reconsideration of their phytosanitary risks.

Introduction

Viroids are the smallest known pathogens of plants, classified in a distinct group of subviral agents. They consist of a single-stranded circular RNA molecule, whose length varies between 246 and 399 nucleotides. *In vitro* viroids are rod shaped because of internal base pairing between the nucleotides. Viroids differ from viruses as they lack a protein shell; in addition, their small genomes do not encode for any protein. Viroids are classified into two families, i.e. the *Avsunviroidae* and the *Pospiviroidae*, consisting of two and five genera, respectively (Flores et al., 2000b). Within the latter family five species from the genus *Pospiviroid* have been isolated from naturally infected tomato (*Solanum lycopersicum*) plants, i.e. *Citrus exocortis viroid* (CEVd; syn. Indian tomato bunchy top viroid, Mishra et al., 1991; Fagoaga & Duran-Vila, 1996), *Potato spindle tuber viroid* (PSTVd, Leontyeva, 1980; Puchta et al., 1990), *Tomato apical stunt viroid* (TASVd, Walter et al., 1980; Walter, 1987), *Tomato chlorotic dwarf viroid* (TCDVd, Singh et al., 1999) and *Tomato planta macho viroid* (TPMVd, Galindo et al., 1982).

Since 1988 the Dutch Plant Protection Service has occasionally detected viroids in diagnostic samples from tomato crops in the Netherlands and from abroad. Although the severity of symptoms varied, infected plants showed chlorosis, bronzing, leaf distortion and growth reduction (Figure 2-1a). Symptoms were most severe for isolate 89001013, which additionally caused a reddening and purpling of the leaves (Figure 2-1b). Infection rates varied from only a limited number of plants up to almost 100% infection. Spreading usually occurred along the rows, indicating



Figure 2-1. Viroid symptoms at naturally infected tomato. (A) Chlorosis, leaf distortion and growth reduction in the top of a tomato plant infected by isolate 95001530. (B) Chlorosis, purpling, leaf curl and growth reduction in a plant infected by isolate 89001013.

that contact during crop handling was the main way of transmission. All infections in the Netherlands were subsequently eradicated.

The presence of the viroids in these symptomatic tomatoes was established by return-polyacrylamide gel electrophoresis (r-PAGE, Huttinga et al., 1987; Roenhorst et al., 2000). However, their identity could not be established by electrophoresis alone. Only the first isolate found in 1988 was additionally sequenced and identified as PSTVd (Puchta et al., 1990). As this isolate (PSTVd-N) considerably differed in sequence from PSTVd isolates sequenced to date, it was considered a new strain. In 1995 PSTVd-N and eight other viroid isolates from tomato were tested by Agdia (Elkart, IN - USA) by hybridisation with DIG-labelled RNA probes for CEVd, *Chrysanthemum stunt viroid* (CSVd), PSTVd, TASVd and TPMVd. Besides isolate N, only one other isolate from tomato reacted with the PSTVd-specific probe. Four isolates hybridised with a probe for CEVd, and the other isolates did not react with any of the probes. This posed the question whether these isolates belong to another of the known species or represent one or more new viroid species.

This chapter describes the characterization of these eight isolates plus five more recently detected isolates by reverse transcription-polymerase chain reaction (RT-PCR) with universal and specific pospiviroid primer sets. Sequence analysis of the amplicons from all thirteen isolates confirmed the positive reactions in the hybridisation tests for PSTVd, but only three of the four positive reactions in the tests for CEVd. Moreover, they revealed the occurrence of TCDVd and *Columnnea latent viroid* (CLVd), a viroid not previously reported in tomato.

Materials and Methods

Viroid isolates

Viroid isolates were obtained from tomato samples submitted for diagnosis to the Plant Protection Service in the Netherlands from 1988 up to 2002. Table 2-1 summarises the tomato varieties affected, the origin of the samples and the estimated infection rates. In addition, PSTVd-H an isolate of PSTVd kindly provided by P. Howell (Scottish Agricultural Science Agency, Edinburgh, Scotland), and PSTVd isolate N (Puchta et al., 1990) were included for reference. After detection by r-PAGE, the isolates were propagated on tomato (see below). Four to five weeks after inoculation young leaves of the inoculated plants were collected and nucleic acids, including viroids, were isolated by a phenol extraction and subsequently stored under ethanol at -20 °C (Roenhorst et al., 2000).

Mechanical inoculation and cultivation of tomato and potato plants

All isolates were mechanically inoculated onto seedlings of tomato cv. Moneymaker as soon as the first true leaves appeared. In addition, isolates PSTVd-N, 89000808, 89001013, 93007481, 93007908 and PSTVd-H were transmitted to potato (*Solanum tuberosum*) cv. Nicola, by mechanical inoculation of the first leaves emerging after planting of the tubers. The inoculated tomato plants were grown for

Table 2-1. Tomato isolates included in this study^a.

Isolate	Tomato variety	Origin	Infection rate (%)	NCBI accession number
89000808	Rondella	NL	<1	AY372390
89001013	Blizzard' + 'Turbo	NL	>90	AY367350
89002594	Dombito	NL	<1	AY372391
89002600	Criterion	NL	<1	AY372393
93007481	Cherry Belle	NL	1-2	AY372392
93007908	Pronto	NL	1-2	AY373446
94005977	Revido	NL	<1	AY372394
95001530	Trust	NL	<1	AY372395
95006685	Cabron	NL	not known	AY372396
96009271	Durintha	Belgium	<1	AY365230
20011470	Daniela ^b	New Zealand	not known	AY372397
21008470	Voyager	NL	<1	AY372398
22006456	Rapsody	USA	30	AY372399
H	Potato	-	-	AY372400
N	unknown	NL	-	X17268

^a PSTVd-N and -H were added as controls

^b variety first showing symptoms

4-6 weeks in a greenhouse under quarantine conditions, with a temperature of 25 °C and supplemental illumination for a day length of at least 14 h. Inoculated potato plants were grown under the same conditions until tubers were produced. Plants were inspected visually twice a week. The same six isolates were also used for mechanical inoculation of potato plants grown in the field under quarantine conditions during two successive years. Ten plants per isolate were inoculated by the end of May and tested by r-PAGE about two months after inoculation.

In 1994, the tubers formed on the inoculated plants in the greenhouse were planted in the field. For three successive years, the tubers harvested from the field were used for planting in the next season. Planting was by the end of April or the beginning of May, and lifting by the second half of July when the haulms of the certified seed potatoes of grade SE had to be killed. For each viroid isolate, ten tubers were planted from which the total weight of the newly-formed tubers was determined after harvest. Storage during the winter season was at 4 °C.

RNA isolation, reverse transcription and polymerase chain reaction (RT-PCR)

For RT-PCR, total RNA was isolated from young tomato leaves (0.5-1.0 g) by using the Pure Script kit (Gentra) according to the manufacturer's instructions. The final RNA pellets were dissolved in 50 µl TE buffer (10 mM Tris, 1 mM Na₂EDTA, pH 8.0) of which 1 µl was used in a one-step RT-PCR (Invitrogen). RT-PCR reactions were performed using primers for the detection of CEVd (Önelge et al., 1997), CSVd (Hooftman et al., 1996), CLVd (Spieker, 1996a) and PSTVd (Shamloul et al., 1997). In addition, two sets of universal primers were used, designed on the basis of an alignment of pospiviroid sequences, i.e. Pospi1-RE (5'-AGC TTC AGT TGT (T/A)TC CAC CGG GT-3'; complementary to nt 261-283) and Pospi1-FW (5'-GGG ATC CCC GGG GAA AC-3'; identical to nt 86-102), and Vid-RE (5'-CCA ACT GCG GTT CCA AGG G-3'; complementary to nt 336-354) and Vid-FW (5'-TTC CTC GGA ACT AAA CTC GTG-3'; identical to nt 355-16); indicated positions refer to NCBI GenBank accession NC_002030 of PSTVd. RT-PCR reactions were performed on a PTC-200 (MJ-Research) thermal cycler programmed for 30 min at 43 °C (cDNA synthesis), 2 min at 94 °C (hot-start activation of *Taq* polymerase), 30 s at 94 °C (denaturation), 1.5 min at 62 °C (annealing), 45 s at 72 °C (elongation), for 15 cycles, followed by 30 cycles with an annealing temperature of 59 °C, and a final extension of 7 min at 72 °C.

PCR products were analysed by electrophoresis through a 2 % agarose gel in TAE buffer containing ethidium bromide (5.0 µg ml⁻¹) and visualised on an UV-transilluminator. An 1 kbp ladder (Invitrogen) was used to estimate sizes of the PCR products.

Sequence and phylogenetic analysis

Sequence analysis of uncloned PCR products was carried out by Eurogentec DNA-sequencing department. The resulting sequences were compared with viroid sequences in the NCBI Genbank using a BLAST (Altschul et al., 1990), and multiple alignments of related sequences were created using Clustal W (Thompson et al., 1994) as implemented on the GENESTREAM network server (www2.igh.cnrs.fr/bin/align-guess.cgi). Minor adjustments were manually introduced in the final alignment to maximise sequence homology.

Phylogenetic relationships among CLVd sequence variants were evaluated using PAUP Version 4.0b10 (Swofford, 1993) and SplitsTree (Huson, 1998). For analyses using PAUP, phylogenetic trees were constructed by exhaustive search. Bootstrap analysis (1000 replicates, Felsenstein, 1985) was performed to estimate support for inferred clades.

Results

Mechanical inoculation of tomato and potato

Under greenhouse conditions, all tomato plants showed growth reduction, and distortion and chlorosis of the young leaves three to five weeks after inoculation.



Figure 2-2. Symptoms appearing on tomato after mechanical transmission of two different viroid isolates. Left non-inoculated; middle: PSTVd-N; right: isolate 89001013.

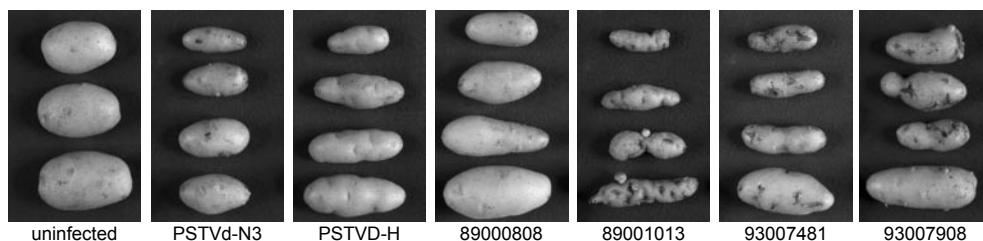


Figure 2-3. Potato tubers showing malformation and various degrees of growth reduction for all six viroid isolates, and additional star cracking for isolates 89001013, 9307481 and 93007908.

Table 2-2. Yield of viroid-infected and healthy potato plants cv. Nicola grown in the field for three successive years^a.

Isolate	1995	1996	1997	Average
PSTVd-N3	2.1 ^b	5.0	0.6	2.6
89000808	5.9	11.7	2.9	6.8
93007481	2.0	3.3	1.4	2.2
93007908	2.6	7.0	0.6	3.4
PSTVd-Howell	7.0	11.2	4.3	7.5
Healthy	14.9	13.6	8.3	12.3

^a In 1994 yield of infected plants was extremely low, because of lack of dormancy of the small tuber harvested in the greenhouse. Plants infected by isolate 89001013 even died within a couple of weeks after emergence without producing any tubers.

^b Total weight (kilograms) of tubers from ten potato plants.

The severity of the symptoms varied between different isolates, isolate 89001013 inducing most pronounced stunting (Figure 2-2).

In the greenhouse the inoculated potato plants did not show any leaf symptoms; tubers of the infected plants were smaller and malformed, however. The severity of the symptoms in potato differed between isolates: isolate 89000808 caused rather mild tuber symptoms, whereas isolates 89001013, 93007481 and 93007908 induced star cracking, severe stunting and malformation (Figure 2-3).

In the field experiments no infections were detected in the inoculated plants by testing in r-PAGE. Potato plants grown from the infected tubers obtained from the greenhouse experiments, however, were severely stunted and their tubers were malformed. Plants infected by isolate 89001013 died within a couple of weeks after emergence, without producing any tubers. Tubers of the other infected plants were cultivated for three successive years. These plants also showed growth reduction and their tubers remained small and often were malformed. Yield losses varied from ca 39% for PSTVd-H up to ca. 82% for isolate 93007481 (Table 2-2).

RNA isolation, reverse transcription and polymerase chain reaction (RT-PCR)

Obvious differences in the severity of the symptoms and the rate of spread in the greenhouses of origin made further characterization of the viroid isolates desirable. First, part of the isolates was tested in RT-PCR by using 'specific' primers for the pospiviroids CEVd, CSVd and PSTVd (Table 2-3). Isolates 89000808, 89002594 and 89002600 produced amplicons with the CEVd primers; PSTVd-H, PSTVd-N3, isolates 9405977, 200011470 and 21008470 produced amplicons with the PSTVd primers. None of the isolates reacted with the CSVd primers. The first set of universal pospiviroid primers (Pospi1-RE/FW) designed to detect all pospiviroids reported in tomato so far, only reacted with those isolates also reacting with the CEVd and PSTVd primers (Table 2-3). Because CLVd was the only pospiviroid not expected to react with the primer set Pospi1-RE/FW, tests were performed with two additional sets of primers: one set specific for CLVd and a second set of universal

Table 2-3. Summary of RT-PCR results with different primer sets.

Isolate	Primer sets for				Designed primer sets	
	CEVd	CLVd	CSVd	PSTVd	Pospi1-RE/FW	Vid-RE/FW
PSTVd-H	-	-	-	+	+	+
N3	-	nt ^a	-	+	+	nt
89000808	+	nt	-	-	+	nt
89001013	-	+	-	-	-	+
89002594	+	nt	-	-	+	nt
89002600	+	nt	-	-	+	nt
93007481	-	+	-	-	-	+
93007908	-	+	-	-	-	+
94005977	-	nt	-	+	+	nt
95001530	-	+	-	-	-	+
95006685	-	+	-	-	-	+
96009271	-	+	-	-	-	+
20011470	-	nt	nt	+	+	nt
21008470	-	nt	nt	+	+	nt
22006456	nt	nt	nt	nt	+	+

^a nt = not tested

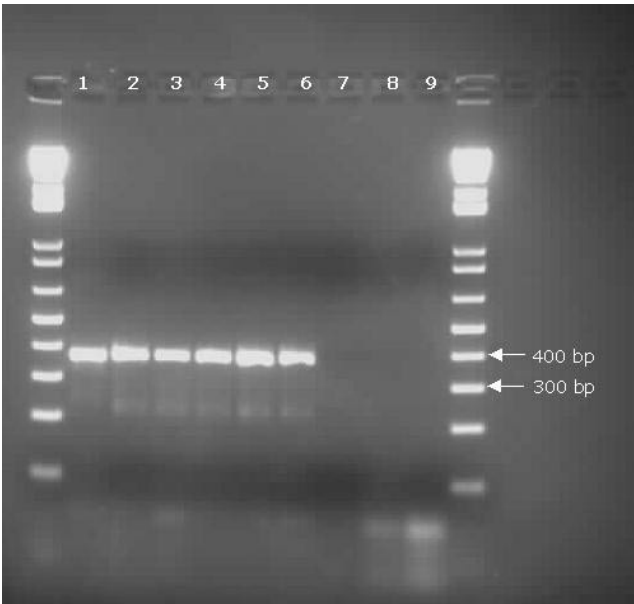


Figure 2-4. Electrophoretic analysis of amplicons yielded by RT-PCR of tomato viroid isolates with CLVd-specific primers: 89001013 (1); 93007481 (2); 93007908 (3); 95001530 (4); 95009685 (5); 96009271 (6); healthy tomato (7); water (8); PSTVd-H (9); 100 bp DNA markers (outer lanes).

primers designed to recognise several pospiviroids including CLVd (Vid-RE/FW). In both tests all isolates previously reacting negative, yielded amplicons of the expected size (Table 2-3, Figure 2-4). Isolate 22006456, which was only tested by Posp1-RE/FW and Vid-RE/FW, yielded amplicons with both primer sets.

Sequence and phylogenetic analysis of PCR amplicons

For all isolates, except PSTVd-N, which already had been sequenced, the amplicons obtained by RT-PCR were directly sequenced, and the resulting sequences deposited in NCBI Genbank (see Table 2-1 for accession numbers). Analysis of the PCR products obtained with the primers for CEVd and PSTVd confirmed their respective identities. The CEVd isolates 89000808, 89002594 and 89002600 showed identities of 92.8-96.0%, 95.8-98.7% and 91.5-93.6% respectively, with the CEVd isolates in the Genbank. In case of PSTVd isolates H, 94005977, 20011470

Table 2-4. Nucleotide identities among three isolates of *Columnea latent viroid* from ornamentals and six viroid isolates from tomato.

Isolate	CLVd-Col	CLVd-Brun	CLVd-Nem	89001013	93007481 ^a	95001530 ^b
CLVd-Col	-	89.9	97.6	89.4	89.9	90.2
CLVd-Brun		-	91.0	84.0	84.5	84.7
CLVd-Nem			-	88.7	90.0	90.3
89001013				-	88.9	89.2
93007481					-	99.7
95001530						-

^a Includes isolates 95006685 and 93007908

^b Includes isolate 96009271

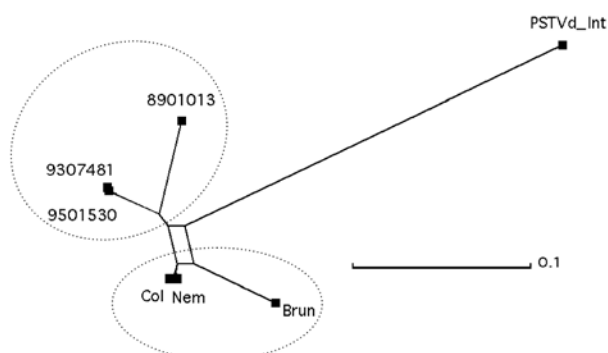


Figure 2-5. Sequence relationships among naturally-occurring isolates of CLVd. Results of SplitsTree analysis, a distance-based method involving Hamming transformation, are shown. Edges of the displayed graph are proportional to the isolation index of the corresponding splits (see scale), and the two lineages are circled. Parsimony and likelihood-based methods predict identical relationships among the six isolates.

and 21008470, identities were 93.6-99.4%, 93.7-94.8%, 93.4-99.4% and 91.6-96.4%, respectively, with the PSTVd isolates in the Genbank. Based on its 96.7% identity with the only TCDVd sequence reported to date, the amplicon obtained from isolate 22006456 with the Vid-RE/FW primers was identified as TCDVd (Singh et al., 1999).

For the remaining six isolates, the amplicons obtained with both the CLVd and the Vid-RE/FW primers were sequenced. As shown in Table 2-4, the resulting sequences appeared most similar to CLVd. Overall identity with CLVd varied between 84.0% and 90.3%, and no sequence changes were present in the central conserved region (CCR) and the terminal conserved region (TCR).

As shown in Figure 2-5, phylogenetic analysis of these sequences indicated that CLVd contains two distinct lineages. The first contains CLVd-Col, isolated from *Columnnea* (Hammond et al., 1989) plus two sequences previously recovered from other ornamental hosts, i.e. *Brunfelsia* (CLVd-Brun, Spieker, 1996a) and *Nematanthus* (CLVd-Nem, Singh et al., 1992a); the second lineage contains the six new isolates from tomato. In each lineage the most divergent isolates are slightly less than 90% identical, i.e. 89.9% for the CLVd-Col and CLVd-Brun versus 88.9% for isolates 89001013 and 93007481. Sequence identity is only 84.0% for the two most divergent isolates from both groups, i.e. the CLVd-Brun and isolate 89001013. Most sequence differences between both lineages were located within the pathogenicity and variable domains.

Discussion

Molecular analysis of the viroids isolated from tomato showed that at least four different viroid species are able to infect this crop. Seven out of the thirteen tomato isolates included in this study belong to viroid species reported in tomato before, i.e. either CEVd (Fagoaga & Duran-Vila, 1996; Mishra et al., 1991), PSTVd (Leontyeva, 1980; Puchta et al., 1990) or TCDVd (Singh et al., 1999). With regard to CEVd, the results of the nucleic acid hybridisation tests performed by Agdia were confirmed for three isolates; isolate 89001013 showed only 61.8% identity with CEVd, however. For PSTVd, isolates 94005977 and 21008470 grouped with the majority of PSTVd isolates in the NCBI Genbank. Isolate 20011470 from New Zealand, however, was most similar to PSTVd-N and isolates described by Behjatnia et al. (1996) and Elliott et al. (2001). All these isolates either originated in or, like the Dutch isolate N (Puchta et al., 1990), could be related to Oceania (i.e. Australia and New Zealand). Finally, 22006456 is the first isolate of TCDVd from tomatoes growing in the USA. The only previous report of TCDVd was from plants growing in Canada (Singh, 1999).

The remaining six isolates were slightly less than 90% identical to two of three published sequences of CLVd, a member of the genus *Pospiviroid* also sharing

some characteristics of the genus *Hostuviroid*. Viroids sharing <90% sequence identity have often been considered to be separate viroid species (Flores et al., 2000b). For these six remaining isolates, indeed, phylogenetic analysis suggests that CLVd-related sequences form two distinct groups/lineages. One group contains the isolates from three ornamental species, i.e. CLVd-Brun, CLVd-Col and CLVd-Nem; the second group contains the six isolates from tomato. Pair-wise sequence identities within each group are slightly less than 90%. Moreover, pair-wise sequence identities between CLVd-Col and CLVd-Nem isolates from the first group and all six tomato isolates from the other, also reveal identities slightly less than 90%. Therefore, the isolates from tomato have been identified as CLVd. Only pair-wise sequences identities between CLVd-Brun and all tomato isolates, respectively, are clearly below 90% (84.0 – 84.7). Since phylogenetic analysis suggests that CLVd-related sequences form two distinct groups/lineages, it is proposed to divide the species CLVd into two strains, i.e. one strain containing the isolates from the three ornamental crops and a second strain containing the isolates from tomato. The fact that like CLVd-Col (Hammond et al., 1989), isolates 89001013 and 93007481 were found to infect cucumber (*Cucumis sativus*, results not shown) further supports their identification as CLVd. It might be worthwhile to determine the susceptibility of cucumber to CLVd-Brun, as the susceptibility of this plant species would substantiate the biological identification of this isolate as CLVd.

Very little is known about the origin of the different viroid infections in tomato. Evidence of a common source has only been obtained for the two PSTVd isolates found in the Netherlands in 1988 (Puchta et al., 1990). At each location, the viroid was identified in pepino (*Solanum muricatum*) as well as in tomato. The pepino plants in these greenhouses were grown from seeds imported both from Greece and New Zealand. These seeds might have provided the pathway for introducing the viroid into the Dutch tomato crops. This hypothesis is supported by the fact that the sequence of the Dutch isolate (PSTVd-N, Puchta et al., 1990) is very similar to the sequences of two isolates from New Zealand, i.e. 20011470 and the one described by Elliott et al. (2001). All these isolates, in turn, closely resemble another PSTVd isolate from Australia (Behjatnia et al., 1996), one that is quite different in sequence from the majority of North American and European PSTVd isolates. More recently, yet other isolates belonging to this group of divergent sequences were reported from tomato in Australia (Mackie et al., 2002) and the United Kingdom (Mumford et al., 2004). For the UK, this was the first natural infection of tomato by PSTVd, but unfortunately its origin could not be traced. Taken together, these observations indicate that this group of aberrant PSTVd isolates may have originated in Oceania.

The possible origins of the other viroid isolates from tomatoes grown in the Netherlands are less clear. The ten viroid infections found between 1989 and 2001, all involved different tomato varieties from five different seed companies. Moreover,

these infections involved three different viroids i.e., CEVd, CLVd and PSTVd. Tracing back the origins of the particular seed lots failed to reveal additional infections. Therefore, it is likely that hosts other than tomato plants may play an important role as source of infection, especially when they fail to show disease symptoms. Natural infections of PSTVd have been found in the solanaceous crops pepino (Puchta et al., 1990; Shamloul et al., 1997), potato (Diener & Raymer, 1971; Martin, 1922) and tomato (Leontyeva, 1980; Puchta et al., 1990) as well as in avocado (*Persea americana*, Querci et al., 1995). The experimental host range of this viroid, however, is quite extensive and includes many more non-solanaceous plants (Singh, 1973). CEVd has been found to naturally infect several plant species (Fagoaga & Duran-Vila, 1996), but TCDVd has only been detected twice in nature – both times in tomato. CLVd infections are symptomless in several ornamental species, i.e. *Brunfelsia undulata* (Spieker, 1996), *Columnea erythrophae* (Hammond et al., 1989) and *Nemanthus wettstenii* (Singh et al., 1992). Such plants provide a likely reservoir for unnoticed viroid spread.

Although isolates of CEVd, CLVd and PSTVd recovered from tomato could be successfully transmitted to potato by mechanical inoculation in the greenhouse, no evidence was found for transmission under field conditions. Field transmission may have been inhibited by the cool growing conditions in the Netherlands, but two months after mechanical inoculation viroid concentrations may also have been too low to be detected by r-PAGE (Manzer & Merriam, 1961; Pfannenstiel & Slack, 1980). Tuber yields varied considerably, but infected tubers were found to produce infected progeny during four successive years. Since growing conditions were not optimal and the size of the experiment was limited, the data on yield reductions should be considered only indicative.

Irrespective of these limitations, the yield reduction data clearly show that CEVd, CLVd and PSTVd have similar effects on potato. The tomato isolates of CLVd even appeared to be more harmful than PSTVd. Experimental transmission of CEVd to potato also has previously been reported by Semancik et al. (1973). Moreover, successful transmission of TCDVd and TPMVd to potato has been also described (Singh et al., 1999; Galindo et al., 1982). Of these five viroids, however, only PSTVd appears in the quarantine lists of the European Union and the European and Mediterranean Plant Protection Organisation. This quarantine status appears mainly based on the risks that PSTVd poses to the seed and ware potato production within its territories. The fact that other viroids can be at least as harmful to potato and tomato as PSTVd stresses the need to reassess the phytosanitary regulations. Moreover, as testing of potato for PSTVd infection previously often was performed by r-PAGE only, it even is possible that some of these other viroids already occur in potato.

Chapter 3

Epidemiological evidence that vegetatively propagated, solanaceous plant species act as sources of *Potato spindle tuber viroid* inoculum for tomato

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Epidemiological evidence that vegetatively propagated, solanaceous plant species act as sources of *Potato spindle tuber viroid* inoculum for tomato Plant Pathology 59, 3-12.

Abstract

In autumn 2006 in the Netherlands *Potato spindle tuber viroid* (PSTVd) infections were detected in 42.3 and 71.9% of professionally grown lots of *Brugmansia* spp. and *Solanum jasminoides*, respectively. The infected lots contained 73,985 and 431,374 plants, respectively, demonstrating the presence of many potential viroid sources for tomato (*Solanum lycopersicum*). PSTVd was identified in cultivars of *B. x candida*, *B. x flava*, *B. sanguinea*, *B. suaveolens*, and unspecified *Brugmansia* species/cultivars. Most infected lots of *Brugmansia* spp. originated from one single Dutch nursery; most infected lots of *S. jasminoides* originated abroad. Sequence analysis revealed that the PSTVd genomes from *Brugmansia* spp. counted an average of 360 nt, whereas all genomes from *S. jasminoides* except one consisted of 357 nt. Furthermore, the collective PSTVd genotypes showed polymorphism at four or more positions, except for two cases in which genotypes from *Brugmansia* spp. and *S. jasminoides* were identical. Phylogenetic studies showed that PSTVd genotypes from *Brugmansia* spp. and *S. jasminoides* grouped apart from each other and from PSTVd isolates from potato (*Solanum tuberosum*) and *Physalis peruviana*. The PSTVd genotypes from tomato did not form a separate cluster but were dispersed over clusters of vegetatively or partly vegetatively propagated plant species, i.e. potato, *P. peruviana* and *S. jasminoides*. Moreover, mechanical inoculation of the predominant PSTVd genotypes from *S. jasminoides* to tomato was successful. These results provide evidence that vegetatively propagated, solanaceous plant species have been sources of infection for tomato crops in the past.

Introduction

Viroids are the smallest known plant pathogens. They only consist of single, circular strands of RNA with a size of 240 - 400 nucleotides, and do not code for any proteins, including a coat protein. This differentiates viroids from plant viruses. The viroids are divided into two families, the *Avsunviroidae* and the *Pospiviroidae*, consisting of three and five genera, respectively. Within the *Pospiviroidae*, the genus *Pospiviroid* contains nine species, with *Potato spindle tuber viroid* (PSTVd) the type species (Flores et al., 2005b).

Since 1988 the Netherlands Plant Protection Service has occasionally detected pospiviroid infections in tomato (*Solanum lycopersicum*) crops from the Netherlands and elsewhere (Verhoeven et al., 2004, 2006a, 2007a). However, attempts to trace the origin of the infections failed in most cases. With the exception of the first two PSTVd infections in the Netherlands, all other infections by *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd) and PSTVd could not be related to specific seed lots and/or nurseries. As CLVd had only previously been reported

from symptomless, vegetatively propagated ornamentals (Hammond, 2003), it was supposed that these plants might have been the sources for infection of the tomato crops (Verhoeven et al., 2004). Therefore, in 2006 a survey for pospiviroids was conducted in ornamental plants (Verhoeven et al., 2008a). Most samples were taken from vegetatively propagated ornamental plants from the families *Gesneriaceae* and *Solanaceae*, the two families including the most known hosts of CLVd and PSTVd. This survey revealed symptomless PSTVd infections in *Brugmansia suaveolens* and *Solanum jasminoides*. PSTVd has a quarantine status in the European Union (EU Directive 2000/29/EC, annex IAI) because it is considered very harmful to potato (*Solanum tuberosum*) and tomato. As a consequence, the introduction and spread of this viroid should be prevented. Therefore, all Dutch growers of *Brugmansia* spp. and *S. jasminoides* were visited to sample and test each lot for PSTVd. If the viroid was detected, all plants of that particular lot were destroyed.

This chapter summarizes the results of the latter survey in the Netherlands, which revealed high infection rates of PSTVd both in *Brugmansia* spp. and *S. jasminoides*, and provided new information on host plants. In addition, a phylogenetic analysis was carried out of sequenced PSTVd genomes from these vegetatively propagated ornamentals, and also potato, Cape gooseberry (*Physalis peruviana*) and tomato. Finally, experiments were conducted to discover whether PSTVd isolates from vegetatively propagated, ornamental plant species were indeed able to infect tomato.

Materials and methods

Sampling

Locations of plants of *Brugmansia* spp. and *S. jasminoides* were traced using growers' records (e.g. delivery notes) and information from auctions. All locations were visited, and 200 random plants per lot were sampled, or all plants when lots contained less than 200 plants. One young full-grown leaf was taken from each plant, and samples were sent in a sealed bag to the laboratories of the Nederlandse Algemene Keuringsdienst voor zaaizaad en pootgoed van landbouwgewassen (NAK) in Emmeloord or the Plant Protection Service (PPS) in Wageningen.

Nucleic acid extraction

Samples were split into subsamples of 25 leaves at most. Per subsample, 1 g of tissue was ground by a Homex grinder (BioReba) in an extraction bag containing 5 ml extraction buffer (0.02 M PBS plus 0.05% Tween, 2% PVP and 0.2% ovalbumine). The RNA was purified with the Pure Script kit (Gentra) according to manufacturer's instructions. The final RNA pellets were dissolved in 40 µl hydration buffer (included in the kit), of which 1 µl was used for real-time RT-PCR (Applied Biosystems) or in a

one-step RT-PCR (Invitrogen).

Real-time RT-PCR and conventional RT-PCR

Real-time RT-PCR was performed according to Boonham et al. (2004), and modified by Roenhorst et al. (2005). Water and PSTVd RNA were included as external controls. Cytochrome oxidase (COX) primers (COX-FW and COX-RE) and probe (COX1511T) were used as internal control (Weller et al., 2000). Conventional RT-PCR was performed using primers 3H1 and 2H1 (Shamloul et al., 1997), in a 25- μ l reaction volume using one-step RT-PCR (Invitrogen) according to manufacturer's instructions.

Sequencing, sequence analysis and phylogenetic studies

Nucleotide sequences of uncloned PCR products were determined by BlaseClear (Leiden, Netherlands). Sequences were analysed by aligning with viroid sequences in the NCBI GenBank using BLASTn (Altschul et al., 1990). Generally, one sequence per lot was determined; however, there were a few cases of *Brugmansia* spp. where no sequencing was performed as the lot originated directly from a lot that had already been sequenced. Evolutionary history was inferred using the Minimum Evolution (ME) method (Rzhetsky & Nei, 1992). Tree no. 1 out of four ME trees (sum of branch length = 0.18469178) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches (Felsenstein, 1985). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki et al., 2004). The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are given as the number of base substitutions per site. To overcome complete gap deletion, sequences were slightly adapted. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) at a search level of 1. The Neighbor-Joining algorithm (Saitou & Nei, 1987) was used to generate the initial tree. Phylogenetic analyses were conducted using MEGA version 4 (Tamura et al., 2007). In addition to the PSTVd genotypes from *Brugmansia* spp. and *S. jasminoides*, previously determined genotypes from potato and tomato were included in these studies. The potato isolates had been intercepted during post-entry quarantine testing of potato breeding material and candidate varieties and originated from various countries. The tomato isolates had been submitted for diagnosis from various countries and were sequenced earlier (Verhoeven et al., 2004, 2007a) or recently. Finally, two PSTVd genotypes from *P. peruviana* originating from Turkey and Germany were included (Verhoeven et al., 2009a).

Mechanical inoculation

Leaf samples of infected *Brugmansia* spp. and *S. jasminoides* plants were inoculated to seedlings of tomato cv. Moneymaker at the first true leaf stage. The predominant PSTVd genotype from *S. jasminoides* (S1) was also inoculated to potato cv. Nicola and *B. suaveolens* cv. Geel plants. The inoculum was prepared according to Verhoeven & Roenhorst (2000). The inoculated tomato plants were grown for 5-6 weeks in a greenhouse under quarantine conditions, at 25 °C with supplemental daily illumination for 13h. Subsequently, plants were tested for the presence of PSTVd by conventional RT-PCR (Shamloul et al., 1997).

Results

PSTVd infections in *Brugmansia* spp.

In *Brugmansia* spp. PSTVd infections were found at 24 out of 29 inspected companies (Table 3-1). Company A and C mainly grew mother plants; A produced young plants for growers, while C mainly produced small quantities of plantlets for hobby growers. Some of the other growers produced marketable plants from their own mother plants, but most plants originated from mother plants of A. At C no PSTVd infections were found in the 294 mother plants (one plant per cultivar). Excluding these 294 plants, 66 (42.3%) of the remaining 156 lots for professional production were found to be infected. These 156 lots contained 145,545 plants of which 73,985 (50.8%) belonged to lots with PSTVd-infected plants.

PSTVd infections were found in at least 21 different cultivars of the following species: *B. x candida*, *B. x flava* (syn. *B. x cordata*), *B. sanguinea*, and *B. suaveolens*. Furthermore, infections were found in samples of unspecified *Brugmansia* species/cultivars, or named as 'mix' or 'yellow/orange' indicating that more than one cultivar was included. None of the tested plants showed viroid symptoms; some leaves - both PSTVd infected and non-infected - showed mosaic as a result of infection by *Colombian datura virus*, a potyvirus commonly found in *Brugmansia* spp. (Lesemann et al., 1996).

The most important source of infection was the Dutch nursery A (Table 3-1) that produced plants for other professional growers. However, other sources of infection might also exist as several growers claimed that they used their own mother plants to produce marketable plants. Furthermore, grower O (Table 3-1) had purchased all plants of three cultivars from a German nursery. The distinctive origin of the three cultivars was further substantiated by the fact that all five PSTVd sequences in these cultivars were identical and belonged to genotype B25, which had not been found in any of the other *Brugmansia* lots. Therefore, it was concluded that grower A was the most important, but not the only source of the PSTVd infections in *Brugmansia* plants in the Netherlands.

Table 3-1. *Potato spindle tuber viroid* infections found in plants of *Brugmansia* spp.

Company ^a	Survey				Origin company A		Origin elsewhere	
	Lots	Positive	Plants	Positive	Lots	Positive	Lots	Positive
A	7	5	260	210	7	5		
B	14	3	43,510	15,500			14	3
C	294	0	294	0			294	0
D	2	2	4,100	4,100	2	2		
E	6	4	488	373	4	4	2	0
F	2	2	450	450	2	2		
G	2	1	70	4	1	1	1	0
H	4	4	81	81	4	4		
I	3	3	120	120	3	3		
J	2	2	500	500	2	2		
K	5	5	222	222	4	4	1	1
L	3	3	285	285	3	3		
M	1	1	6,600	6,600	1	1		
N	1	1	400	400	1	1		
O	3	3	3,540	3,540			3	3
P	1	1	11,000	11,000	1	1		
Q	2	0	3,500	0			2	0
R	61	4	22,254	6,091			61	4
S	1	1	157	157			1	1
T	3	2	2,035	1,035			3	2
U	4	1	9,600	800			4	1
V	5	2	20,511	9,500			5	2
W	1	0	1	0			1	0
X	1	0	78	0	1	0		
Y	3	1	7,000	6,000			3	1
Z	1	1	2	2			1	1
AA	1	0	1	0			1	0
BB	8	6	3,795	2,115	5	5	3	1
CC	9	8	4,985	4,900	9	8		
Total	450	66	145,839	73,985	50	46	400	20

^a letters for companies in Table 3-1 and 3-2 do not correspond

PSTVd infections in *S. jasminoides*

In *S. jasminoides* PSTVd infections were found at 38 out of 51 companies inspected (Table 3-2). The viroid was identified in 69 of 96 lots tested (71.9%). None of the tested plants showed viroid symptoms. The lots had no cultivar names; some lots were indicated by the colour of the flowers as white or blue. The total number of

Table 3-2. *Potato spindle tuber viroid* infections found in plants of *Solanum jasminoides*.

Company ^a	Lots	Positive	Plants	Positive	Company ^a	Lots	Positive	Plants	Positive
A	1	0	5,200	0	AA	1	1	10,000	10,000
B	1	1	2,000	2,000	BB	1	1	70	70
C	1	1	5,000	5,000	CC	2	2	48,100	48,100
D	2	0	81	0	DD	1	0	100	0
E	1	1	1,900	1,900	EE	1	1	600	600
F	1	1	5,100	5,100	FF	2	2	2,830	2,830
G	1	1	1,800	1,800	GG	1	0	10,000	0
H	1	1	12,000	12,000	HH	1	1	1,500	1,500
I	2	2	12,600	12,600	II	1	0	4,000	0
J	2	1	2,100	2,000	JJ	7	7	31,500	31,500
K	2	2	1,050	1,050	KK	3	3	4,500	4,500
L	1	1	5,500	5,500	LL	3	1	57,000	15,000
M	2	0	260	0	MM	1	1	20,000	20,000
N	1	0	900	0	NN	2	2	160	160
O	2	2	41,000	41,000	OO	1	1	28,800	28,800
P	2	1	11,150	150	PP	1	0	78	0
Q	5	4	60,600	31,600	QQ	1	0	600	0
R	4	4	18,800	18,800	RR	5	1	43,800	15,000
S	1	0	3,000	0	SS	2	1	43	7
T	2	2	30,600	30,600	TT	1	1	1	1
U	1	0	27	0	UU	8	8	46,600	46,600
V	1	0	200	0	VV	1	1	247	247
W	2	1	10,000	9,500	XX	2	0	10,000	0
X	2	2	15,028	15,028	YY	1	1	984	984
Y	2	2	4,600	4,600	ZZ	1	1	247	247
Z	2	2	5,000	5,000	Total	96	69	577,256	431,374

^a letters for companies in Table 3-1 and 3-2 do not correspond

lots comprised 577,256 plants, of which the infected lots represented 431,374 plants (74.7%). In addition to the infections found at the Dutch nurseries, PSTVd was also identified in three lots of *S. jasminoides* imported from Israel and in two illegally imported consignments from Kenya.

PSTVd infection in *Datura* sp.

In addition to the PSTVd infections in *Brugmansia* spp. and *S. jasminoides*, PSTVd was identified in a single plant of *Datura* sp.. This plant was one of approximately 400 that had been raised from seed. Five of these *Datura* plants had been grown among PSTVd-infected plants of *B. suaveolens* cv. Wit; one of

these plants was infected. The other *Datura* plants had been grown in the same greenhouse separated from the infected *Brugmansia* plants, and none of them was infected.

Tomato chlorotic dwarf viroid (TCDVd) infection in *B. sanguinea* cv. Oro Verde

Testing of the 294 lots of *Brugmansia* from grower C revealed the presence of TCDVd in a single plant of *Brugmansia sanguinea* cv. Oro Verde. The viroid reacted positive both in real-time and conventional RT-PCR and was identified by sequence analysis of the full-length amplicons obtained by the latter method. The nucleotide sequence was deposited in the NCBI GenBank (Accession No EF626530). The viroid isolate was successfully transmitted to young tomato plants to prove pathogenicity. The discovery of TCDVd here shows the need for sequence analysis of PCR products for pospiviroid identification.

Sequence analysis of PSTVd isolates

For *Brugmansia* spp., 68 PSTVd sequences were analysed. The sequences could be classified in 32 different genotypes, designated B1 for the isolate from *B. suaveolens* first sequenced (Verhoeven et al., 2008a; NCBI GenBank Accession No. EF192394) up to B32 (Figure 3-1). Genotype B1 was found most frequently, i.e. in 20 isolates (29.4%) from 13 companies (Figure 3-2A). In addition, genotypes B2, B18, B23, B24, B25, B29 and B30 were found four, five, three, two, five, three and two times, respectively. Genotypes B24 and B25 were found at only one company each, while the five remaining genotypes were found at two or three companies. All other genotypes were only identified once. The sequences had an average length of 360 nt, but varied between 357 and 363 nt. Three genotypes (B2, B18 and B28) only differed by one nucleotide substitution from genotype B1. Most isolates revealed polymorphism at two to seven nucleotide positions; however, isolates B4 and B16 showed 10 polymorphic sites each. The high sequence variation may be related to high genetic variation in the infected plants of *Brugmansia*, i.e. at least 21 cultivars belonging to four plant species. Furthermore, it indicates that the infections may be long-standing.

For *S. jasminoides*, sequence analysis of 82 isolates only revealed four different genotypes. The genotypes were classified as S1 for the isolate first sequenced (Verhoeven et al., 2008a; NCBI GenBank Accession No. EF192393) up to S4 (Figure 3-1). Genotype S1 was found most frequently, i.e. in 57 isolates

Figure 3-1. (Page 47) Multiple sequence alignment of *Potato spindle tuber viroid* genotypes from *Brugmansia* spp. (B1-B32) and *S. jasminoides* (S1-S4). B1 is the reference genotype first isolated from *Brugmansia suaveolens* (NCBI GenBank Accession Number EF192394); S1, marked grey, is identical to the previously submitted sequence EF192393 (Verhoeven et al., 2008a). Nucleotide identity to B1 is represented by dots, deletions by -, and insertions and substitutions by letters representing the nucleotides. Note for B25, S1, S2 and S4 the substitution A→U at position 121, the final nucleotide of the Central Conserved Domain, and the three deletions at positions 124, 127 and 238.

B1	CGGACUAAUCUGUGUGUUCUUGUGUUCACACUGAGCU	CC	.UGAGCGAGAAAGAAAAGAAUGUGCGGCGGCGUCGAGAGGCGUUCAGGGAUCCCGGAGAAACUUGAGCGAGCAUCUGCGAAAGAGACUGGCGAGAGUCCGAGCGCGACAGAGTAUUCCGCGAGAACAGGGUUU	B1
B2	B2
B3	U	B3
B4	U.C	B4
B5	GAAG	B5
B6	U..C	B6
B7	U..	B7
B8	U..C	B8
B9	U..	B9
B10	U..	B10
B11	U..	B11
B12	U..	B12
B13	U..	B13
B14	U..	B14
B15	U..	B15
B16	U..	B16
B17	U..	B17
B18	U..	B18
B19	U..	B19
B20	U..	B20
B21	U..	B21
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B27	U..	B27
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B30	U..	B30
B31	U..	B31
B32	U..	B32
B33	U..	B33
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B36	U..	B36
B37	U..	B37
B38	U..	B38
B39	U..	B39
B40	U..	B40
B41	U..	B41
B42	U..	B42
B43	U..	B43
B44	U..	B44
B45	U..	B45
B46	U..	B46
B47	U..	B47
B48	U..	B48
B49	U..	B49
B50	U..	B50
B51	U..	B51
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B315	U..	B315
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B318	U..	B318
B319	U..	B319
B320	U..	B320
B321	U..	

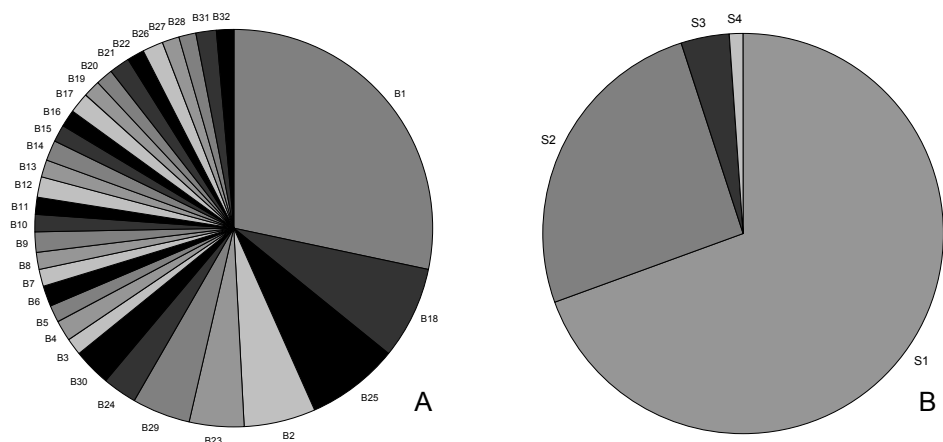


Figure 3-2. Genetic variation in *Potato spindle tuber viroid* (PSTVd) genotypes from *Brugmansia* spp. and *Solanum jasminoides*. (A) The 68 sequenced PSTVd isolates from *Brugmansia* spp. belonging to 32 different genotypes; all occurring at low frequencies (1-5), except for genotype B1 (20). (B) The 82 sequenced PSTVd isolates from *S. jasminoides* belonging to four different genotypes, S1 (57) and S2 (21) occurring at high frequencies.

(69.5%) from 29 companies (Figure 3-2B). In addition, genotype S2, with only a single nucleotide substitution, was found in 21 isolates (25.6%) from 16 companies. The remaining genotypes, S3 and S4, were only found in three and one isolates, respectively. Genotype S1 also was detected four times in imported plants from Israel and Kenya, while S2 was found once in plants from Israel. The sequences had a length of 357 nt, except for S3 that consisted of 360 nt. S2 and S4 each only expressed one single substitution relative to S1, but S3 had three insertions and four substitutions.

Comparison of the predominant genotypes from *Brugmansia* spp. (B1) and *S. jasminoides* (S1) showed substantial differences, i.e. four substitutions and three indels. Mutual comparison of the other genotypes from *Brugmansia* spp. and *S. jasminoides* generally showed higher discrepancies. The exceptions were B18, which showed one substitution less, B20, which showed the same number of substitutions and indels, and B23 and B25, which were identical to S3 and S1, respectively. Furthermore, the PSTVd genotype from the single infected plant of *Datura* sp. appeared identical to genotype B1 from *Brugmansia* spp.; this genotype was also identified from *B. suaveolens* cv. Wit in the same glasshouse, which suggests that the viroid was transmitted from *B. suaveolens* cv. Wit to the *Datura* plant.

Phylogenetic studies

All PSTVd genotypes from *Brugmansia* spp. that were found more than once and all genotypes from *S. jasminoides* were included in the phylogenetic study. However, B25 and S3 were excluded because they were identical to S1 and B23, respectively. PSTVd genotypes from potato, tomato and *P. peruviana* were

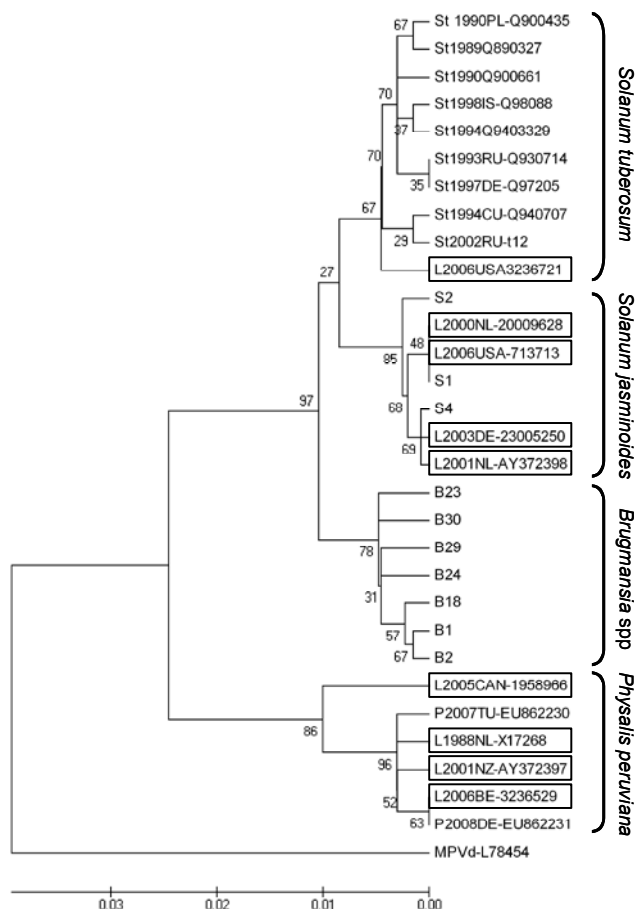


Figure 3-3. Evolutionary relationships of 31 *Potato spindle tuber viroid* PSTVd genotypes from *Brugmansia* spp., *Solanum jasminoides*, *Physalis peruviana*, potato and tomato. Other phylogenetic methods produced similar trees. PSTVd isolates from *Brugmansia* spp., *S. lycopersicum*, *P. peruviana*, *S. jasminoides* and *S. tuberosum* start with B, L, P, S and St, respectively. The letters B and S are followed by their genotype number; L, P and St are followed by the year of sample submission, the country of the origin (when known), a hyphen, and letter/figures for genotype identification. PSTVd genome clusters from vegetatively propagated plant species are braced; individual PSTVd genomes from tomato are boxed.

also included. The genotypes grouped in four clusters (Figure 3-3). Each cluster included genotypes from one vegetatively propagated plant species, i.e. potato, *S. jasminoides*, *Brugmansia* spp. and *P. peruviana* (a vegetable crop propagated both by cuttings and seeds, and a perennial in the tropics). The PSTVd genotypes from seed-propagated tomato were dispersed over all clusters, except that containing the genotypes from *Brugmansia* spp.. Four tomato isolates grouped in the cluster of isolates from *P. peruviana*, a cluster of PSTVd genotypes mainly found in Oceania and already recognized as separate from the other PSTVd genotypes (Puchta

Table 3-3. Results of mechanical inoculation of *Potato spindle tuber viroid* isolates from *Brugmansia* spp. (B genotypes) and *Solanum jasminoides* (S genotypes) to tomato cv. Moneymaker; successful inoculations were confirmed by RT-PCR.

Genotype	Inoculations	Successful	Non-successful
B1	6	1	5
B3	1	0	1
B5	1	0	1
B6	1	0	1
B7	1	0	1
B8	1	0	1
B9	1	0	1
B16	1	1	0
B17	1	0	1
B24	1	0	1
B25 (= S1)	5	0	5
B28	1	0	1
S1	7	5 ^a	2
S2	1	1 ^a	0
Total	29	8	21

^a sequencing of PCR products showed no mutations in the viroid genome



Figure 3-4. Tomato cv. Moneymaker plant showing stunting and mild chlorosis 5 weeks after inoculation with *Potato spindle tuber viroid* genotype S2.

et al., 1991; Verhoeven et al., 2004). The remaining five PSTVd genotypes from tomato grouped in the PSTVd genotype clusters from potato (one genotype) and *S. jasminoides* (four genotypes), two of the latter group even being identical to genotype S1.

Mechanical inoculation

Most PSTVd isolates from *S. jasminoides*, but only few from *Brugmansia* spp., were successfully transmitted to tomato cv. Moneymaker (Table 3-3). Tomato plants infected by isolates from *Brugmansia* spp. and *S. jasminoides* showed only very mild symptoms and mild to intermediate symptoms, respectively (Figure 3-4). Sequencing of S1- and S2-inoculated tomato plants showed no nucleotide sequence mutations compared to the inoculated genotype. The plants of potato cv. Nicola and of *B. suaveolens* cv. Geel inoculated with PSTVd genotype S1 from *S. jasminoides* still did not show symptoms after 8 weeks. However, testing of the inoculated plants by RT-PCR using primers 3H1/2H1 (Shamloul et al., 1997) revealed that all five potato plants and three out of six plants of *B. suaveolens* cv. Geel were infected. Moreover, sequencing of uncloned PCR products showed that S1 still was the predominant PSTVd genotype in the plants of both potato and *B. suaveolens* cv. Geel.

Discussion

The results of the current survey and data from previously sequenced PSTVd genotypes support the hypothesis that vegetatively propagated, ornamental plants species have been sources of PSTVd infections in tomato in the Netherlands (Verhoeven et al., 2004). Furthermore, the results show that also the tropical perennial *P. peruviana*, which is propagated vegetatively as well as by seed, may also have been a source of infection. These conclusions are based on four observations: 1) the previously performed back-tracing of pospiviroid infections in tomato, 2) the high PSTVd infection rates found in *Brugmansia* spp. and *S. jasminoides*, 3) the phylogenetic grouping of PSTVd genotypes from tomato with those from vegetatively propagated plant species, and 4) the results of 'natural' and experimental transmissions of specific PSTVd genotypes.

The back-tracing of pospiviroid infections in tomato in the Netherlands (Verhoeven et al., 2004) and Belgium (Verhoeven et al., 2007a) provided no evidence that tomato seeds and tomato plants from nurseries had been the sources of the infections. From 1988 up to 2007 three pospiviroid species were identified in thirteen examined infections of tomato, and in all cases plants belonged to different varieties from five different seed companies. When infections of the same viroid were found in the same year, the plants were never raised in the same nursery. Therefore, it was concluded that seeds and plants of tomato were not the sources for these infections.

The recent survey in *Brugmansia* spp. and *S. jasminoides* showed that PSTVd was present in many lots, consisting of 10 to hundreds of thousands of plants, respectively. A number of factors may have contributed to these high infection rates. First, PSTVd-infected plants of both crops are symptomless and as a consequence, infected plants may have already been used for propagation over a long period. Secondly, both crops are propagated by cuttings, which is an efficient way to transmit viroids. Thirdly, international trade of ornamental plants has increased enormously during the last decade. Together, these factors may have contributed to a rapid spread all over the world. This was demonstrated by the infected plants of *S. jasminoides* found in consignments from Israel and Kenya. In addition to our findings in *Brugmansia* spp. and *S. jasminoides* in the Netherlands, the presence of PSTVd in these crops has also been reported from other European countries (Anonymous, 2008; Di Serio, 2007), as well as in other vegetatively propagated plant species, i.e. *Lycianthes rantonnetii*, syn. *S. rantonnetii* (Di Serio, 2007), *P. peruviana* (Verhoeven et al., 2009a), and *Streptosolen jamesonii* (Verhoeven et al., 2008b).

The phylogenetic study showed that PSTVd genotypes from vegetatively propagated plant species group apart, while the genotypes from seed-propagated tomato grouped with genotypes from *P. peruviana*, *S. tuberosum* and *S. jasminoides*, respectively (Figure 3-3). As infections in vegetatively propagated crops are maintained continuously, these plants may serve as permanent sources of infection for other plants. In contrast, seed-propagated tomato plants need to be infected every season unless seeds are continuously produced from infected plants. Considering the serious symptoms of PSTVd in tomato, seed production on infected plants is expected to happen only rarely and, therefore, tomato seeds are not likely to act as a permanent source of infection. Hence, the phylogenetic relations between PSTVd genotypes from vegetatively propagated plant species and tomato suggest that the vegetatively propagated plant species have been the main sources of infection for the tomato crops considered.

The phylogenetic study also indicated that PSTVd infections in *S. jasminoides* date back several years, because the PSTVd genotypes concerned have been identified in tomato since 2000 (Figure 3-3). A partially sequenced Australian tomato isolate from 2001 (Hailstones et al., 2003) can probably be added to the tomato isolates grouping with the isolates from *S. jasminoides* because the sequenced 311 nt of this isolate show highest identities with the PSTVd genotypes in this cluster. Furthermore, genotypes identical to the ones reported here in *S. jasminoides* from the Netherlands, Israel and Kenya have been reported in this species and the vegetatively propagated *L. rantonnetii* in Italy (Di Serio, 2007), and *S. jamesonii* from Israel (Verhoeven et al., 2008b). Despite the high infection rates in these ornamentals, however, the low number of outbreaks caused by these PSTVd genotypes in tomato since 2000 shows that the viroid had been transmitted from these ornamentals to tomato only sporadically.

The number of PSTVd isolates from tomato grouping with the isolates from *P. peruviana* in our phylogenetic study could also be extended. Based on their nucleotide sequence, tomato isolates from Australia (Behjatnia et al., 1996; Mackie et al., 2002), New Zealand (Elliott et al., 2001) and the United Kingdom (Mumford et al., 2004) all belong to the 'oceanian' strain of PSTVd isolates. As *P. peruviana* is cultivated and grows in the wild in Australia, this species may have been the source of infection for the PSTVd outbreaks in tomato in that country.

The genotypes from seed-propagated tomato grouped with genotypes from *P. peruviana*, *S. tuberosum* and *S. jasminoides*, respectively, but not with genotypes from *Brugmansia* spp. (Figure 3-3). This might be explained by the lower number of infected plants of *Brugmansia* spp. than of *S. jasminoides* (Table 3-1 and 3-2). In addition, the mechanical transmission experiments showed that PSTVd transmission from *Brugmansia* spp. to tomato appeared more difficult than from *S. jasminoides* to tomato and, therefore, may have happened less frequently. The poor mechanical transmission from *Brugmansia* leaves may have been caused by a high content of inhibitors.

Finally, our hypothesis that vegetatively propagated plant species were the sources of PSTVd infections in tomato was supported by various transmission data. Mechanical inoculations of S1- and S2-PSTVd isolates from *S. jasminoides* frequently resulted in successful transmission to tomato cv. Moneymaker (Table 3-3), S1 and S2 still being the predominant genotypes. PSTVd from *P. peruviana* was also successfully transmitted to tomato (Verhoeven et al., 2009a). Further evidence for PSTVd transmission from vegetatively propagated ornamentals is provided by the discovery of B25 at a single location (grower O) in three *Brugmansia* cultivars, all originating from the same foreign nursery. B25 is quite aberrant from the other PSTVd genotypes from *Brugmansia* spp. (Figure 3-1), having three deletions and four nucleotide substitutions, one of which is even located at the final nucleotide position 121 (A→U) of the Central Conserved Domain (CCD; Keese & Symons, 1985). However, B25 is identical to S1, the genotype commonly found in *S. jasminoides*. Therefore, it is assumed that this genotype B25 (S1) originated from plants of *S. jasminoides*, which were commonly infected by this genotype. This is further supported by the fact that in the mechanical-inoculation experiment, genotype S1 from *S. jasminoides* was shown to infect *B. suaveolens* cv. Geel without any sequence changes. A similar explanation can be given for S3/B23: S3 is also quite aberrant from the other PSTVd genotypes from *S. jasminoides* in having three insertions at positions that are unanimously found in the PSTVd sequences from *Brugmansia*, and by a substitution (U→A) at the final position of the CCD that is also found unanimously in the sequences from *Brugmansia* spp.. Further indications for transmission of PSTVd between plant species are provided by the identifications of S1 in *L. rantonnetii* (Di Serio, 2007), B1 in *B. suaveolens* cv. Wit and *Datura* sp., and S2 in both *S. jasminoides* and *S. jamesonii* (Verhoeven et al., 2008b).

This study provides comprehensive evidence that vegetatively propagated, solanaceous plant species have been sources of infections for PSTVd in tomato in the past. However, the question remains how the viroid was transmitted from these plants to tomato. In general, four means of viroid transmission exist: vegetative propagation, seeds/pollen, humans and insects. Both vegetative and seed propagation can be excluded because these only account for transmission within the same plant species. Therefore, transmission by humans and insects have to be considered.

Transmission of PSTVd by contact during crop handling has been reported in potato and tomato crops (Manzer & Merriam, 1961; Verhoeven et al., 2004). In tomato this transmission pathway is assumed to be the predominant one as the viroid mainly spreads along rows. Alternate handling of infected and non-infected plants of different plant species in the same glasshouse without taking hygienic measures may transmit the viroid from one species/cultivar to another. PSTVd was probably transmitted between the different ornamental species in this way. In this study PSTVd was mechanically transmitted from *Brugmansia* spp. and *S. jasminoides* to tomato under experimental conditions. The question remains, however, how frequently this will happen in practice where ornamental plants are usually grown far apart from tomatoes.

Insects may also play a vector role, as reported for aphids, and for PSTVd transmission by transencapsidation the ornamental plants would need to be coinfecting by *Potato leafroll virus* (Querci et al., 1997; Syller et al., 1997). In addition, Antignus et al. (2007) recently reported transmission in tomato of the related *Tomato apical stunt viroid* (TASVd) by bumble bees. However, it is not clear whether such transmission would also be effective between plant species. Furthermore, the question arises whether thrips could transmit PSTVd by mechanical inoculation of infected pollen as reported for ilarviruses (Sdoodee & Teakle, 1993). Therefore, investigations should be carried out on the role of thrips and bumble bees in the transmission of PSTVd from ornamental plant species to potato and tomato, respectively.

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Chapter 4

High stability of original predominant pospiviroid genotypes upon mechanical inoculation from ornamentals to potato and tomato

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Abstract

Eleven pospiviroid isolates from ornamental plants and one from pepper were successfully transmitted to potato and tomato by mechanical inoculation. All isolates with characteristic predominant genotypes were inoculated to a series of potato and tomato plants and propagated for up to four passages. In total, 385 nucleotide sequences were determined, in which 17 new predominant genotypes were identified with minimal differences to the original predominant genotype. In addition, in the original ornamental hosts only one of eleven predominant pospiviroid genotypes had changed during the experiments over a period of two years. These results confirm the high stability of predominant pospiviroid genotypes.

Introduction

Viroids and most plant viruses have RNA genomes. These genomes show the highest mutation rate among living species, i.e. 10^{-3} to 10^{-5} errors per nucleotide per replication cycle versus 10^{-8} to 10^{-11} for DNA genomes because RNA polymerases lack proof reading and repair mechanisms (Eigen & Biebricher, 1988; Gago et al., 2009; Holland et al., 1982). In addition, both viroids and plant viruses have very small and compact genomes, short generation times and extremely large populations (Domingo & Holland, 1997). This together allows fast adaptation of genomes to changing conditions by selection of beneficial mutants. The balance between continuous generation and consecutive selection of mutants leads to a dynamic population structure, known as 'quasi-species' (Domingo & Holland, 1997; Keese et al., 1988). Viroids propagate in their plant hosts as quasi-species, with one genotype usually dominating the population in the case of the family *Pospiviroidae* (Martínez-Soriano et al., 1996; Sanjuán et al., 2006), i.e. the predominant genotype or master sequence, and more sequence variation in the case of the family *Avsunviroidae* (Ambros et al., 1998; De la Peña et al., 1999).

Sequencing of uncloned PCR products from plants of *Brugmansia* spp. infected by *Potato spindle tuber viroid* (PSTVd) revealed many different predominant genotypes, whereas in *Solanum jasminoides* only a few predominant sequences were found (Verhoeven et al., 2010). Generally, a single predominant genotype was present in each plant or group of plants tested because the obtained sequences did not show ambiguity. Also for the newly characterized *Pepper chat fruit viroid* (PCFVd) sequencing of cloned PCR products revealed a single predominant genotype (Verhoeven et al., 2009b). In this chapter the genetic variability is studied, i.e. the combined action of mutation and selection (Gago et al., 2009), of the predominant genotypes of five pospiviroids upon transmission from ornamental plants and pepper (*Capsicum annuum*) to potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*).

Materials and Methods

Mechanical inoculation

Leaves of naturally infected plants (Table 4-1) were mechanically inoculated to young plants of potato cv. Nicola (4-6 leaves) and tomato cv. Moneymaker (1-2 true leaves) in five replications according to Verhoeven & Roenhorst (2000). For *Brugmansia* spp. roots were used as inoculum source because inoculation with leaf sap was often unsuccessful. Inoculated plants were grown at 25 °C with supplemental daily illumination for 13 h. From all inoculated plants, leaf tissue was collected 6 weeks after inoculation for RT-PCR and sequencing and stored at –20 °C for 2-4 months. After sequencing, for each predominant genotype frozen leaf tissue was inoculated to the next series of five potato and tomato plants, respectively. In total, each original predominant genotype was inoculated 4 times; newly arisen predominant genotypes were inoculated 0-3 times depending on the passage number, at which they were encountered.

RT-PCR and sequencing

For RT-PCR 1 g of young leaves per plant was ground by a Homex grinder (BioReba) in an extraction bag containing 5 ml buffer (0.02 M PBS plus 0.05% Tween

Table 4-1. Sources of infections and primers used for RT-PCR amplification.

Predominant viroid genotype	Isolate	Plant species	Reference ^a	Primers ^b	Reference
CEVd-S1	3823889	<i>Solanum jasminoides</i>	5	CEVd-FW/RE	1
PCFVd-C1	3259237	<i>Capsicum annuum</i>	6	AP-FW1/RE2	6
PSTVd-B1	3153387	<i>Brugmansia suaveolens</i> 'Light Red'	7	3H1/2H1	2
PSTVd-B1	3153311	<i>Brugmansia suaveolens</i> 'Wit'	7	3H1/2H1	2
PSTVd-B11	3153395	<i>Brugmansia x flava</i> 'Orange'	7	3H1/2H1	2
PSTVd-B34	3241168	<i>Brugmansia x candida</i> 'Variegata'	8	3H1/2H1	2
PSTVd-S1	3077695	<i>Solanum jasminoides</i>	7	3H1/2H1	2
PSTVd-S2	3497501	<i>Streptosolen jamesonii</i>	4	3H1/2H1	2
PSTVd-S2	3002685	<i>Solanum jasminoides</i>	7	3H1/2H1	2
TASVd-C1	3153272	<i>Cestrum</i> sp.	3	CEVd-FW/RE	1
TCDVd-B1	3816013	<i>Brugmansia sanguinea</i> 'Oro Verde'	7	3H1/2H1	2
TCDVd-P1	Q06383	<i>Petunia</i> sp.	9	3H1/2H1	2

^a 1 = Önelge, 1997; 2 = Shamloul et al., 1997; 3 = Verhoeven et al., 2008a; 4 = Verhoeven et al., 2008b; 5 = Verhoeven et al., 2008c; 6 = Verhoeven et al., 2009b; 7 = Verhoeven et al., 2010; 8 = NCBI GenBank Accession Number GQ396665; 9 = NCBI GenBank Accession Number GQ396664

^b Primer pair CEVd/FW/RE and 3H1/2H1 amplified the complete viroid genome; primer pair AP-FW1/RE2 amplified the complete genome except for a fragment of 13 nt

plus 2% PVP, MW 10,000 plus 0.2% ovalbumine). The RNA was purified using the Masterpure kit (Epicentre) according to manufacturer's instructions and dissolved in 40 µl hydration buffer (included in the kit). One microliter was used in a one-step RT-PCR using RT / Platinum® *Taq* mix (Invitrogen) according to manufacturer's instructions and using the primers listed in Table 4-1. Nucleotide sequences of uncloned PCR products were determined by BaseClear, Leiden, the Netherlands and analysed by aligning with the sequences of the predominant viroid genotypes from the original host plants using Clustal W (Thompson et al., 1994). The sequence of the predominant genotypes in the original hosts was determined up to 6 months before the start of the experiments and after completion (ca. 2 years later) for those plants still available.

Results

All original predominant genotypes were successfully transmitted to both potato and tomato, except for *Citrus exocortis viroid* (CEVd)-S1 (Table 4-2). Inoculation with this genotype resulted in a single mutation (position 227: G→C) in all nine successfully inoculated plants and an additional mutation (position 6: U→A) in one of the potato plants (Table 4-3). This indicates a probable misreading or misinterpretation of the original CEVd-S1 genotype in *S. jasminoides*. Furthermore, PSTVd-B34 was transmitted only at low frequencies during all four passages.

In total, the predominant sequences were determined in 385 infected potato and tomato plants (Table 4-2). In potato and tomato, eleven and six changes, respectively, were recorded in the predominant sequences, excluding the four plants of both potato and tomato infected by CEVd-S1a after the first passage. Some changes were stable and were transmitted at high rates, e.g. PSTVd-B1a and *Tomato chlorotic dwarf viroid* (TCDVd)-P1a; others were not transmitted and were lost, e.g. PSTVd-B34a and b, and CEVd-S1b (Table 4-2 and 4-3). In general, a new predominant genotype was found in only one of the inoculated plants and had only a single substitution or deletion (Table 4-3). Insertions were not observed. Only the changes resulting in genotypes PSTVd-B34a and TCDVd-P1c consisted of multiple substitutions or deletions. In addition to the change CEVd-S1→S1a, only PSTVd-S2 → S1 showed the same change in more than one plant; i.e. three of five inoculated potato plants showed the substitution U→A at position 221 after the third passage. As a result, the predominant position of genotype S2 was taken over by S1, the genotype most frequently found in *S. jasminoides* (Verhoeven et al., 2010). After the fourth passage the situation was unstable; i.e. both genotypes were competing for dominance since sequencing of the viroid from tomato showed approximately equal peaks in the electropherograms at position 221 for the nucleotides A (S1) and U (S2), and sequencing from potato showed dominance for S1 twice and S2 once.

For the original predominant genotypes 0 to 4 new predominant genotypes were found. In the most frequently found PSTVd genotype, B1 from *Brugmansia* spp., only one change in predominant genotype was encountered. Moreover, no changes were found in PSTVd genotype S1, the most frequently found genotype in *S. jasminoides* (Verhoeven et al., 2010). This indicates that these common genotypes maintained their dominance better than genotypes B11 and B34, which were found in *Brugmansia* spp. only once. In addition, no changes were observed in the predominant genotypes PCFVd-C1 and *Tomato apical stunt viroid* (TASVd)-C1, confirming the dominance of the genotype PCFVd-C1 (Verhoeven et al., 2009b) and indicating a high dominance of TASVd-C1. The dominance of genotype TCDVd-P1 from *Petunia* sp. changed most frequently, i.e. four times; in contrast, dominance of the TCDVd isolate from *B. sanguinea* 'Oro Verde' was more stable since only one new predominant genotype was discovered. Furthermore, some mutations were identified more frequently than others. Preferring PSTVd mutations include: 65 U→G (genotype B11b and B34a), 162 C→U (B34a and B34c), 223 A→U (B1, reverted from B1a, and B34a), all of which are reversions to the predominant PSTVd sequence B1 from *Brugmansia* spp. (Verhoeven et al., 2010), and 258 U→A (B11a and B34b).

With regard to the stability of the pospiviroid genomes in the original host plants, it was found that PSTVd-B1 (both isolates), PSTVd-B11, PSTVd-S1, PSTVd-S2 (1 isolate), TASVd-C1, TCDVd-B1, and TCDV-P1 were still predominant at completion of the experiments after 2 years; only the predominant genotype PSTVd-B34 had changed to B34b. The original *S. jasminoides* plants infected by CEVd-S1 and PSTVd-S2 (isolate 3002685), respectively, and *C. annuum* infected by PCFVd-C1, were no longer available at the end of the experiments.

Discussion

The results presented in this paper show that pospiviroid genotypes from various ornamentals and pepper are able to infect potato and tomato. This means that these often symptomless-infected ornamental plants pose a risk to economically important crops, which may result in serious diseases. The intriguing question remains why only such a limited number of pospiviroid infections in tomato, and none in potato, have been reported during the last decade, despite the presence of large numbers of infected ornamental plants (Verhoeven et al., 2010). This might be due to low chances for transmission - the inoculum source often is not present in the immediate vicinity of tomato - or to poor transmission rates as observed for leaves of *Brugmansia* spp. (Verhoeven et al., 2010).

B34 was included in the experiments because in most of the previous experiments we were not able to obtain a predominant sequence in the infected *B.*

Table 4-2. Evolution of the inoculated genotypes along the subsequent passages.

Pospiviroid	Genotype	Isolate	Length	Potato: passage number				Tomato: passage number			
				1 ^a	2	3	4	1	2	3	4
	CEVd-S1	3823889	374	0/5*				0/4*			
	CEVd-S1a		374	4**/5	2/3	1/1	3/5	4**/4	2/2	2/3*	4/4
	CEVd-S1b		374	1**/5	0/0				ni ^c		
	CEVd-S1c		374				0/2			1**/3	5/5
	PCFVd-C1	3259237	348	5/5	4/5	4/4	5/5	5/5	2/2	0/0	2/5
	PSTVd-B1	3153387	360	4/5	1/1	4/5*	5/5	5/5	4/4	5/5	4/4
	PSTVd-B1a		360			1**/5	4/5				3/4* ^b
	PSTVd-B1	3153311	360	4/4	2/2	2/2	4/4	3/3	2/2	5/5	4/4
	PSTVd-B11	3153395	361	1/2*	0/0	1/1	3/4*	5/5	2/2	5/5	4/4
	PSTVd-B11a		361	1**/2	1/2	1/1	0/0		2/2	5/5	4/4
	PSTVd-B11b		361				1**/4				
	PSTVd-B34	3241168	361	1/2	0/0	1/1	0/2*	0/3*	1/1	2/2	2/2
	PSTVd-B34a		361		0/0			1**/3	0/0		
	PSTVd-B34b		361		0/0			1**/3	0/0		
	PSTVd-B34c		361				1**/2				

Pospiviroid Genotype	Isolate	Length	Potato: passage number				Tomato: passage number			
			1 ^a	2	3	4	1	2	3	4
PSTVd-S1	3077695	357	5/5	5/5	4/4	5/5	5/5	5/5	4/4	5/5
PSTVd-S2	3497501	357	4/4	5/5	2/5*	4/4	5/5	4/5	5/5	5/5
PSTVd-S2a	357				3**/5	3 ^d /3				5 ^e /5
PSTVd-S2	3002685	357	ni	ni	3/4	4/4	5/5	2/2	2/5	5/5
TASVd-C1	3153272	365	5/5	0/1	5/5	5/5	5/5	1/3	5/5	5/5
TCDVd-B1	3816013	356	2/3	4/4	4/4	1/1	4/4	2/2	5/5	3/4*
TCDVd-B1a	355									1**/4
TCDVd-P1	Q06383	359	5/5	4/4	3/5*	4/4	5/5	3/5*	5/5	5/5
TCDVd-P1a	359				4/5	2/2		2**/5	5/5	4/5
TCDVd-P1b	358				2**/5	1/2*				0/1*
TCDVd-P1c	357									1**/1
TCDVd-P1d	357					1**/2				
Total			42/45	28/32	45/47	56/62	53/54	34/37	56/59	71/76

^a a number of predominant sequences determined / number of successful inoculations

^b back-mutation from PSTVd-B1a to PSTVd-B1 in one of four plants

^c ni = not inoculated

^d S1 was identified twice; S2 once

^e sequence ambiguity (A/U) at position 221

* passage in which substitutions of the predominant viroid genotype were observed

** number of plants in which mutations were observed

Table 4-3. Recorded mutations in pospiviroid genomes upon transmission to potato and tomato.

Original genotype	Isolate	Sequences analysed	Mutations in original genotype			Mutations in mutants					
			#/host ^a	Passage	Genotype	Location(s)	#/host	Passage	Genotype	Location(s)	Mutated genotype
CEVd-S1	3823889	29	4/p	1	S1a	227 G→C (4x)	1/t	3	S1c	303 A→U	S1a
			4/t	1	S1a	227 G→C (4x)					
			1/p	1	S1b ^b	6 U→A; 227 G→C					
PCFVd-C1	3259237	27	-				-				
PSTVd-B1	3153387	40	1/p	3	B1a	223 U→A	1/t	4	B1	223 A→U	B1a
PSTVd-B1	3153311	26	-				-				
PSTVd-B11	3153395	36	1/p	1	B11a	65 U→G	-				
			1/p	4	B11b	258 U→A					
PSTVd-B34	3241168	10	1/t	1	B34a ^b	65 U→G; 162 C→U; 223 A→U	-				
			1/t	1	B34b ^b	258 U→A					
			1/p	4	B34c	162 C→U					
PSTVd-S1	3077695	38	-				-				
PSTVd-S2	3002685	21	-				-				
PSTVd-S2	3497501	44	3/p	3	S1	221 U→A	1/p	4	S2	221 A→U	S1
							5/t	4	S1 or S2	221 A and U	
TASVd-C1	3153272	31	-				-				
TCDVd-B1	3816013	26	1/t	4	B1a	179 -A	-				
TCDVd-P1	Q06383	56	1/p	2	P1a	195 G→U	1/p	4	P1c	4 A→G; 332 -U	P1b
			1/p	3	P1b	196 -A	1/t	4	P1d	181 -C	P1b

^a a number of substituted predominant sequences / plant species in which mutation was observed: p = potato; t = tomato
^b mutant was not transmitted to potato and tomato in the subsequent passage

candida 'Variegata' plant. This indicates that more genotypes were competing for dominance, which is substantiated by the fact that a different predominant genotype, B34b instead of B34, was found in the original host at the end of the experiment. Moreover two mutants, B34a and B34b, were found after the first passage, each in one tomato plant, while the original B34 sequence was still found in one potato plant. Neither mutant could be further transmitted to potato and tomato, whereas the original B34 was only transmitted occasionally (Table 4-2). This indicates that genotype B34 and its mutants are not very capable of infecting potato and tomato. In contrast, the most frequently identified PSTVd genotype in *Brugmansia* spp. B1 (Verhoeven et al., 2010) only showed a single mutation, which even reverted to the original sequence in one of five inoculated tomato plants after subsequent passage.

The low genetic variability (Gago et al., 2009) recorded in our experiments is in line with previous data. The PSTVd isolate from *Solanum commersonii* sequenced by Van Wezenbeek et al. (1982) has been used as a positive control in our routine viroid tests since 1981. This isolate has been maintained by many repeated inoculations on tomato over 27 years. Sequencing of this isolate in 2002 (Verhoeven et al., 2004) and 2007 (results not shown) revealed just one single insertion (A) at the position 54. Once infection was established in potato, Owens et al. (2009) also observed low sequence diversity after characterizing 39 PSTVd isolates collected over a 15-year period from widely separated areas in Russia. These results may be explained by the combination of the large-scale use of PSTVd infected in vitro plants and a low genetic variability.

The finding of low genetic variability and predominant pospiviroid genotypes also corresponds to results obtained by Codoñer et al. (2006) with *Chrysanthemum stunt viroid* (CSVd) but contrast to their results with *Chrysanthemum chlorotic mottle viroid* (CChMVd). This may be explained by the fact that the viroids included in our study belong to the same family - and even genus - as CSVd, whereas CChMVd is a member of the family *Avsunviroidae*. *In silico* studies by Sanjuán et al. (2006) showed more mutational effects for the members of the *Avsunviroidae*. These were explained by larger fragility of the multi-branched secondary structures formed by the members of the *Avsunviroidae* in comparison with the rod-like structures of the *Pospiviroidae*. As an alternative, the different mutation frequencies between members of both viroid families may be explained by a presumed lower fidelity of the chloroplastic RNA polymerase used by the *Avsunviroidae* in comparison with the nuclear RNA polymerase used by the *Pospiviroidae*. Finally, our experiments were performed under favourable conditions - including suitable host plants - for viroid replication, which may have contributed to a relatively low mutation rate. Such conditions stimulate the survival of the fittest instead of the survival of the flattest (Codoñer et al., 2006; Elena & Sanjuán, 2007), and thus favoured the predominance of a single genotype. Therefore, we assume that the low variability showed by the sequences of the uncloned PCR products in our experiments support the hypothesis

that pospiviroids in solanaceous plants display a quasi-species formed by one single predominant genotype that may be accompanied by additional genotypes at low frequencies.

The conclusion that the substitution rate of predominant pospiviroid genotypes is low when pospiviroids are transferred between solanaceous crops concurs with that of Owens (2008). In addition, it implies that the predominant genotypes from naturally infected host plants may be used for phylogenetic analysis to trace sources of infection. As a consequence, it supports the phylogenetic analyses performed by Verhoeven et al. (2010), which revealed that PSTVd infections in tomato were related to infections in *S. jasminoides* and *Physalis peruviana*, with these latter crops functioning as sources of infection. Moreover, the low substitution rates indicate that host-plant adaptation of viroid genomes as recorded by Verhoeven et al. (2010) for *Brugmansia* spp, *P. peruviana*, *S. jasminoides* and *S. tuberosum* needed a long time.

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Chapter 5

Mechanical transmission of *Potato spindle tuber viroid* between plants of *Brugmansia suaveolens*, *Solanum jasminoides*, potato and tomato

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“Mechanical transmission of *Potato spindle tuber viroid* between plants of
Brugmansia suaveolens, *Solanum jasminoides*, potato and tomato”.

Abstract

Potato spindle tuber viroid (PSTVd) has been found in many solanaceous ornamental plant species recently. This study reports on the effectiveness of four ways of mechanical transmission between *Brugmansia suaveolens*, *Solanum jasminoides* potato (*Solanum tuberosum*) and tomato (*Solanum esculentum*). Three ways were successful, i.e. inoculation with 'infected' plant sap diluted in water, by fingertips and razor blades. Temperature, plant species to be inoculated and source of inoculum were found critical factors in transmission. An average temperature of 15 °C appeared critical for successful inoculation but inoculations were more successful at 20 and 25 °C. In addition, tomato was more susceptible to PSTVd than *Brugmansia suaveolens*, *Solanum jasminoides* and potato. Furthermore, *S. jasminoides* was a better source of inoculum than *B. suaveolens*. No transmission was obtained after repeated adding of inoculum to tomato roots. These results indicate that PSTVd can be transmitted by crop handling between plant species in practice.

Introduction

Viroids are the smallest infectious agents of plants known. They consist of a circular single-stranded RNA molecule with a size between circa 250 and 400 nt (Diener 1999; Flores et al., 2005a) that does not encode for any proteins. This means that viroids totally rely on the machinery of their host plants for replication and for transport within and to other plants.

The currently characterized viroid species have been assigned to eight genera in two families, the *Avsunviroidae* and the *Pospiviroidae* (Flores et al., 2005b). *Potato spindle tuber viroid* (PSTVd) is one of the nine species assigned to the genus *Pospiviroid* of the latter family. The recently identified *Pepper chat fruit viroid* has been nominated as the tenth member of this genus (Verhoeven et al., 2009b). As the type member of this genus PSTVd has been studied extensively because of both its scientific and economic importance (Owens, 2007; Owens & Verhoeven, 2009). In potato (*Solanum tuberosum*) yield and economic losses date back to the early 20th century. In addition, over the last decades occasional outbreaks of PSTVd and other pospiviroids in tomato (*Solanum lycopersicum*) have been reported (Leontyeva, 1980; Puchta et al., 1990; Verhoeven et al., 2004).

Pospiviroids can be transmitted in different ways, by vegetative propagation, mechanical transmission, true seed and pollen, and by insects. In the case of vegetative propagation tubers, cuttings and micro-plants from infected plants produce permanent sources of infection for other lots and crops. Vegetative propagation is considered to account for the majority of spread of PSTVd in potato and PSTVd and other pospiviroids in ornamentals like *Brugmansia* spp. and *Solanum jasminoides*

(Owens et al., 2009; Owens & Verhoeven, 2009; Verhoeven et al., 2010). Moreover, the absence of symptoms in ornamentals increases the risk of propagating infected plants. Mechanical transmission also has been found to account for pospiviroid spread; although, efficiency of transmission may vary considerably (Manzer & Merriam, 1961; Hollings & Stone, 1973). Evidence for this way of transmission is provided by the observation that pospiviroids spread along rows in pepper (*Capsicum annuum*), potato and tomato (Owens & Verhoeven, 2009; Verhoeven et al., 2004 and 2009b). Transmission by true seed and pollen has been reported as the third way of transmission (Hunter et al., 1969; Krycynski et al., 1988; Singh, 1970). In this way PSTVd is assumed to have spread among potato germplasm collections all over the world. True seed may also contribute to spreading in generatively propagated crops like pepper and tomato. Many pospiviroid infections in these crops, however, could not be related to infected seed lots (Verhoeven et al., 2004, 2007a). Finally, transmission by insects has been reported for a few viroids. Aphid transmission has been reported for PSTVd in potato as a result of transencapsidation of viroid RNA by the coat protein of *Potato leafroll virus* (Querici et al., 1997; Syller et al., 1997) and for *Tomato planto macho viroid* (TPMVd) in tomato (Gallindo et al. 1986). Transmission by bumble bees has been reported for *Tomato apical stunt virus* (TASVd) (Antignus et al., 2007), and *Tomato chlorotic dwarf viroid* (TCDVd) (Matsuura et al., 2010). In the latter cases the mechanism of viroid transmission is not clear, yet.

The fact that the pospiviroid infections in tomato in the Netherlands could not be related to infected seed lots raised questions on the origin of the respective viroids (Verhoeven et al., 2004). Together with the recent identification of different pospiviroids in various ornamental plant species (Di Serio, 2007; Nie et al., 2005; Singh et al., 2006; Verhoeven et al., 2008a, 2008b), an association between these infections has been suggested. Evidence that the infections in tomato indeed originated from ornamentals has been provided by comparison of the sequences of PSTVd genomes from ornamentals and tomato (Navarro et al., 2009; Verhoeven et al., 2010). How the PSTVd isolates had been transmitted from ornamentals to tomato, however, was still unknown. Transmission by vegetative propagation and true seed could be excluded, since these ways only enable transmission within a plant species. Therefore, the question arose if mechanical transmission, including pollen (Sdoodee & Teakle, 1993), and/or insects could account for the transmission from ornamentals to tomato. This chapter reports on the efficiency of PSTVd transmission by crop handling within *B. suaveolens* and *S. jasminoides* and from these crops to potato and tomato.

Materials and Methods

Inoculum sources

For inoculation we used PSTVd genotype B1 from *B. suaveolens* cv. Light Red and S1 from *S. jasminoides* (Verhoeven et al., 2008a and 2010). Both isolates were maintained by propagating the originally infected plants in a quarantine greenhouse at common temperatures for these crops, i.e. 21 °C and 18 °C during day and night, respectively, and with additional light for 13h during daytime. For the trial of mechanical inoculation by watering of plants both inoculum sources were grown in a quarantine-growing chamber at 25 °C, 70% humidity and 16/8h day/night photoperiod.

Cultivation of plants before inoculation and stage of inoculation

Potato cv. Nicola was raised from eye-plugs; tomato cv. Sheyenne and cv. Moneymaker were raised from seeds and *B. suaveolens* cv. Geel and *S. jasminoides* cv. Variegata from cuttings. Absence of viroid has been assured by testing. All plants were grown under conditions as described before.

Mechanical inoculation by diluted sap of infected plants and by contaminated fingertips

Potato and tomato cv. Sheyenne plants were inoculated by diluted plant sap and by contaminated fingertips when they had 4-6 and 3-4 true leaves, respectively. For inoculation with diluted sap, inocula were prepared by grinding 1 g of leaves from inoculum sources in water (Milli-Q). Previous experiments had shown higher PSTVd concentrations in water than in buffer during various periods of storage at 20 °C (results not shown). Moreover, also in practice no chemicals are added for viroid preservation. For each inoculum a 10-fold dilution series was prepared from 10^{-1} (1 g leaf tissue/10 ml) to 10^{-5} . In quantitative RT-PCRs (Boonham et al., 2004) these diluted inocula produced Ct-values from 21.2 to 31.2 for *B. suaveolens* cv. Light Red, and from 22.0 to 30.7 for *S. jasminoides*. All inoculum dilutions were mechanically rubbed on carborundum-dusted leaves of five potato and tomato plants.

Fingertips were contaminated by rubbing young leaves of the inoculum source between thumb, index and middle finger. One minute, 10 minutes and 2 hours after acquiring the viroid, a group of five potato or tomato plants was inoculated by smoothly rubbing non-carborundum-dusted leaves. Within the groups of five plants the order of inoculation was always the same. Between virus acquisition and inoculation the contaminated fingers did not touch any materials. After the last inoculation the fingers were cleaned thoroughly by washing with soap. A single person only inoculated one inoculum per 24 hours.

Inoculated potato and tomato plants were grown in two groups at constant

temperatures of 15 °C and 25 °C for 6 weeks, simulating the average temperature per 24-hours period during the growing season of seed potatoes, i.e. 15 °C, and a good temperature for viroid replication/multiplication, i.e. 25 °C (Verhoeven&Roenhorst, 2010). Three mock-inoculated potato and tomato plants were grown as controls. The experiment was performed in two replications.

Watering of plants with inoculum

Tomato plants cv. Sheyenne having 2-3 true leaves were transplanted to pots with 40 ml of silicate sand (0.1-1.3 mm) and grown in a quarantine-growing chamber. The inocula for PSTVd-B1 and -S1 were prepared daily by grinding 0.5 g leaf tissue in 10 ml RNase-free water (Milli-Q). Plants were inoculated by adding 2 ml of inoculum to the soil via a pipette without touching the plants; after 2-4 hours 3-4 ml nutrient solution (Flora series solution, 1 ml in 1000 ml water) was added in the same way. For both PSTVd-B1 and -S1 8 plants were inoculated, 4 plants for 5 consecutive days and 4 plants for 10 days. After the final watering plants were transplanted separately to a Klasman Surfinia/Carilon substrate.

Mechanical inoculation by contaminated razor blades

Plants of *B. suaveolens* cv. Geel, *S. jasminoides* cv. Variegata and tomato cv. Moneymaker were inoculated with PSTVd-contaminated razor blades at plant heights between 10 and 20 cm. To acquire the viroid leaves and stems of each inoculum source were cut by a razor blade 8 to 10 times. For inoculation tops of a group of 5 healthy plants were cut. Per plant species the groups of five plants were inoculated once, twice or three times at a time interval of 2 weeks. Per group, the viroid was only acquired before inoculation of the first plant. Within the groups the order of inoculation was always the same. For all plants that were inoculated once, one razor blade was used per inoculum; for the second and third inoculation a new razor blade was used per group of five plants in order to prevent cross-contamination between groups. The experiment was performed in three replications and per species three plants were mock inoculated. Plants were grown under conditions as described for inoculum sources of *B. suaveolens* and *S. jasminoides*.

Checking inoculated plants for PSTVd infections

To identify infected plants symptom expression was unreliable. Potato plants, *B. suaveolens* and *S. jasminoides* did not show leaf symptoms, and symptoms on both tomato cultivars appeared erratically and often were dubious. Therefore, all inoculated plants were tested for the presence of PSTVd by real-time RT-PCR (Boonham et al., 2004), those inoculated with diluted plant sap or contaminated fingers 6 weeks and all other plants 8 weeks after the (last) inoculation.

Results

The inoculation of diluted sap was unsuccessful for nearly all inoculations at 15 °C. Only a few tomato plants became infected with PSTVd-S1 when the inoculum was diluted no more than 100-fold (Table 5-1). At 25 °C the inoculations were more successful, and even inoculum dilutions up to 10⁻⁵ resulted in infection when inoculating tomato with PSTVd-S1. Using *B. suaveolens* as source of infection, inoculations were only successful using a 10-fold sap dilution. In addition, potato was less susceptible to PSTVd-S1 than tomato.

Comparable results were obtained using the fingertip-inoculation method (Table 5-2). At 15 °C only a single tomato plant became infected by PSTVd-S1. At 25 °C all tomato plants became infected by this isolate and 25 out of 30 plants by PSTVd-B1. If not all five plants per series became infected, the infected plants were randomly spread in the series. For the fingertip-inoculation method potato appeared less susceptible to PSTVd than tomato but using PSTVd-S1 still resulted in infections up to ten minutes after rubbing the sap of the infected plants onto the fingertips.

Adding PSTVd-contaminated water to the growing medium of tomato seedlings did not cause any infection, irrespective the viroid isolate or the frequency of inoculation.

Inoculation by PSTVd-contaminated razor blades was very successful for tomato. With the inoculum PSTVd-S1 12 out of 15 tomato plants became infected

Table 5-1. Diluted-sap inoculation of PSTVd from *Brugmansia suaveolens* and *Solanum jasminoides* to potato cv. Nicola and tomato cv. Sheyenne using 10-fold inoculum dilutions at 15 and 25 °C.

Dilution ^a	Inoculum for <i>Brugmansia suaveolens</i> (B1)				Inoculum from <i>Solanum jasminoides</i> (S1)			
	Potato	Tomato			Potato	Tomato		
15 °C	I ^b	II	I	II	I	II	I	II
10 ⁻¹	0 ^c	0	0	0	0	0	5	0
10 ⁻²	0	0	0	0	0	0	4	0
10 ⁻³	0	0	0	0	0	0	0	0
10 ⁻⁴	0	0	0	0	0	0	0	0
10 ⁻⁵	0	0	0	0	0	0	0	0
25 °C	I	II	I	II	I	II	I	II
10 ⁻¹	4	4	4	4	4	5	5	5
10 ⁻²	0	0	0	0	0	1	5	5
10 ⁻³	0	0	0	0	0	2	5	5
10 ⁻⁴	0	0	0	0	0	0	5	3
10 ⁻⁵	0	0	0	0	0	0	3	4

^a Leaf sap was diluted in 10-fold steps

^b I = first replication; II = second replication

^c number of infected plants from 5

after a single slash with a contaminated razor blade; 2 or 3 slashes resulted in infection of all inoculated plants (Table 5-3). Using PSTVd-B1 was a little less successful but still the majority of the inoculated plants became infected after 2 and 3 slashes. If not all five plants per series became infected, infected plants were randomly spread in the series. In contrast to tomato, viroid spread within the ornamentals was very low: no infection was detected in plants of *S. jasminoides* cv. Variegata, and only a single plant of *B. suaveolens* cv. Geel became infected after three slash-inoculations with PSTVd-S1. Remarkably, this was the last of the five *Brugmansia* plants of a series that was inoculated with the same razor blade. The results from the inoculations by both fingertips and razor blades show that transmission may be erratic and infectivity persists outside plants up to hours.

Table 5-2. Fingertip inoculation of PSTVd from *Brugmansia suaveolens* and *Solanum jasminoides* to potato cv. Nicola and tomato cv. Sheyenne using three different latency periods at 15 and 25 °C.

Latency ^a	Inoculum from <i>Brugmansia suaveolens</i> (B1)				Inoculum from <i>Solanum jasminoides</i> (S1)			
	Potato		Tomato		Potato		Tomato	
15 °C	I ^b	II	I	II	I	II	I	II
1 min	0 ^c	0	0	0	0	0	0	0
10 min	0	0	0	0	0	0	1	0
2 h	0	0	0	0	0	0	0	0
25 °C	I	II	I	II		II	I	II
1 min	0	0	5	5	0	3	5	5
10 min	0	0	1	5	0	5	5	5
2 h	0	0	5	4	0	0	5	5

^a Leaf sap was diluted in 10-fold steps

^b I = first replication; II = second replication

^c number of infected plants from 5

Table 5-3. Single, double and threefold razor-blade inoculation of PSTVd from *Brugmansia suaveolens* and *Solanum jasminoides* to *B. suaveolens* cv. Geel, *S. jasminoides* cv. Variegata and tomato cv. Moneymaker.

Slashes	Inoculum from <i>Brugmansia suaveolens</i> (B1)			Inoculum from <i>Solanum jasminoides</i> (S1)		
	<i>B. suaveolens</i>	<i>S. jasminoides</i>	<i>S. lycopersicum</i>	<i>B. suaveolens</i>	<i>S. jasminoides</i>	<i>S. lycopersicum</i>
1	0 ^a	0	4 (0-3)	0	0	12 (3-5)
2	0	0	11 (3-4)	0	0	15
3	0	0	9 (1-5)	1 (0-1)	0	15

^a total number of infected plants of three replications (maximum 15); range within numbers of infected plants per replication between parentheses

Discussion

The results of the transmission experiments showed that PSTVd is transmitted successfully by traditional mechanical inoculation and by contaminated fingertips and razor blades. The efficiency of transmission varied and appeared clearly influenced by temperature. At 15 °C only a few tomato plants became infected, whereas many plants became infected at 25 °C. For tomato these results confirm those of Grasmick & Slack (1985) and Sanger & Ramm (1975). For potato inoculation at 15 °C was unsuccessful. This might explain the lack of transmission during previous field experiments in the Netherlands (Verhoeven et al., 2004), since the average temperature per 24-hours period during the growing season of seed potatoes was 15.1 °C for the period 1901-2008 (KNMI-daggegevens, n.d.). Since Singh & Dilworth (2009) had shown that TCDVd survived temperatures of -12 °C in *Vinca minor* our results indicate that 15 °C may be too low for adequate replication of the viroid, which substantially reduces the chance for successful inoculation.

The results from both the traditional mechanical inoculation using a dilution range and the fingertip inoculation show that tomato is more susceptible to PSTVd than potato. In contrast, Singh et al. (1990) found transmission of PSTVd to potato at a twofold lower viroid level (calculated as 0.12 ng/ml) than to tomato. However, Grasmick & Slack (1985) had reported even successful transmissions to tomato after inoculating with 0.56×10^{-3} ng/ml down to 0.56×10^{-7} ng/ml PSTVd. In our study we did not only demonstrate that transmission of lower PSTVd concentrations was more successful in tomato than potato but we also showed that transmission by contaminated fingertips after long latency periods was more efficient for tomato than potato.

With regard to the inoculum source, PSTVd-S1 from *S. jasminoides* was transmitted more successful than PSTVd-B1 from *B. suaveolens*. This is rather due to the host plant than to the PSTVd genotype. Also in previous experiments poor transmission rates were obtained when using leaves of *Brugmansia* spp. as sources of PSTVd inoculum (Verhoeven et al., 2010). In these experiments five out of seven mechanical inoculations with PSTVd-S1 from *S. jasminoides* were successful, whereas all five inoculations with the same genotype from *Brugmansia* spp. were unsuccessful. Since three genotypes of PSTVd and one of *Tomato chlorotic dwarf viroid* (TCDVd) were transmitted successfully from roots of *Brugmansia* spp. (Verhoeven & Roenhorst, 2010), an unknown leaf component is presumed to inhibit viroid transmission. In the case of fingertip inoculation, this negative effect on viroid transmission is less pronounced, probably because the leaves are less damaged in this case.

The fingertip inoculation appeared very successful for transmitting PSTVd from ornamentals to tomato. Seigner et al. (2008) already showed successful

transmission after a short latency period with a PSTVd isolate from *S. jasminoides*. Our experiments show that infectivity may even be maintained over a longer period of time, as high infection rates were found even after latency periods of 10 minutes and 2 hours. This would enable mechanical viroid transmission from ornamentals to potato and tomato in practice, even if these plant species are not grown in each others' vicinity. It should be considered, however, that under natural conditions, contaminated fingers touch other materials during the latency period, which may effect the transmission efficiency.

The finding that PSTVd was transmitted very efficiently from *B. suaveolens* and *S. jasminoides* to tomato via contaminated razor blades shows that contaminated knives and scissors may form a way for viroid transmission in practice. Within ornamental plants transmission by contaminated razor blades hardly occurred in our experiments. This indicates that this way of transmission is not very efficient in *B. suaveolens* and *S. jasminoides*. For *Citrus*, however, several viroids have been reported to be transmitted by contaminated knives at low frequencies (Barbosa et al., 2005). Therefore, the role of viroid transmissions via contaminated knives and shears should not be neglected because in some crops, like *S. jasminoides*, plants may be cut very frequently for taking cuttings and pruning.

Seigner et al. (2008) did not observe transmission after adding PSTVd one time into pots with healthy tomato plants. We showed that repeated adding of inoculum to the rooting medium of tomato plants did not result in viroid transmission, either. These results indicate that in tomato crops the spreading of pospiviroids along the rows (Verhoeven et al., 2004) is not via soil or hydroponic systems but most likely results from crop handling.

In summary, our finding that PSTVd is transmitted successfully from ornamentals to tomato by contaminated fingertips and razor blades supports the hypothesis that ornamentals formed the origin of viroid infections in tomato in the past (Verhoeven et al., 2004, 2010). For potato the lack of transmission of PSTVd at 15 °C might explain that outbreaks in this crop have not been observed in the Netherlands, so far. Moreover, they indicate that under the field conditions in the Netherlands primary infections in seed potatoes are unlikely to occur.

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Chapter 6

Pepper chat fruit viroid: biological and molecular properties of a proposed new species of the genus *Pospiviroid*

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Abstract

In autumn 2006, a new disease was observed in a glasshouse-grown crop of sweet pepper (*Capsicum annuum*) in the Netherlands. Fruit size of the infected plants was reduced up to 50%, and plant growth was also slightly reduced. Here we show that the disease is caused by a previously non-described viroid. The pepper viroid is transmitted by both mechanical inoculation and pepper seeds and, when inoculated experimentally, it infects several solanaceous plant species inducing vein necrosis and reduced fruit and tuber size in tomato and potato, respectively. The viroid RNA genome consists of 348 nucleotides and, with minor modifications, it has the central conserved and the terminal conserved regions characteristic of members of the genus *Pospiviroid*. Classification of the pepper viroid within the genus *Pospiviroid* is further supported by the presence and structure of hairpins I and II, the presence of internal and external RY motifs, and phylogenetic analyses. The primary structure of the pepper viroid only showed a maximum of 66% nucleotide sequence identity with other viroids, which is far below the main species demarcation limit of 90%. According to its biological and molecular properties, we propose to assign the pepper viroid to a new species within the genus *Pospiviroid*, and to name this new species *Pepper chat fruit viroid*.

Introduction

Viroids are small circular single-stranded RNAs of approximately 245-400 nucleotides (nt) that are able to infect plants (Diener, 1999; Flores et al., 2005a). They share structural similarities with certain satellite RNAs; however, viroids replicate autonomously whereas replication of satellite RNAs depends on their helper viruses. Viroids do not code for any protein, and thus they lack the characteristic capsid of viruses. Taxonomically, viroids are divided into the families *Pospiviroidae* and *Avsunviroidae*, consisting of five and three genera, respectively. Within the family *Pospiviroidae*, the nucleotide sequence of the central conserved region (CCR), in combination with the nucleotide sequence of the terminal conserved region (TCR) or the terminal conserved hairpin (TCH), have been used for genus discrimination (Flores et al., 1997; Koltunow & Rezaian, 1988). Species demarcation criteria include sequence identity below 90% and different biological properties, particularly host range and symptom expression (Flores et al., 2005a). Currently, 29 viroid species have been admitted by the International Committee on Taxonomy of Viruses (ICTV), six are listed as tentative viroid species (Flores et al., 2005b), and another two have been characterized recently (Nakaune & Nakano, 2008; Serra et al., 2008).

In the autumn of 2006, a new disease was observed in a glasshouse-grown crop of sweet pepper (*Capsicum annuum*) in the Netherlands. The type of symptoms

and the distribution pattern in the glasshouse raised the suspicion of a virus or viroid infection. However, symptoms differed not only from those incited by known viruses, but also from *Potato spindle tuber viroid* (PSTVd), the only known viroid reported from *C. annuum* (Lebas et al., 2005). Therefore, studies were started to detect and identify the viruses and viroids in the diseased pepper plants. This chapter reports results showing that the disease is caused by a viroid. Biological and molecular properties of the pepper viroid support that it should be considered a new species of the genus *Pospiviroid*, for which we propose the name *Pepper chat fruit viroid* (PCFVd) on the basis of symptom analogy (reduced fruit size) to apple chat fruit disease (Luckwill, 1963).

Materials and Methods

Mechanical inoculation

For biological studies, plant leaves were mechanically inoculated according to Verhoeven & Roenhorst (2000). Unless otherwise indicated, inoculated plants were grown at 25 °C with supplemental daily illumination for 13 h over 6 weeks. To fulfill Koch's postulates, after return-polyacrylamide gel electrophoresis (r-PAGE) (Roenhorst et al., 2000; Schumacher et al., 1986), nucleic acids were eluted from the position of a non-stained polyacrylamide gel where viroid RNA was expected to migrate and then inoculated into pepper plants cv. Yolo Wonder. These plants were tested 6 weeks later for the presence of the viroid by RT-PCR using the primer pair AP-FW1/RE2 (Table 6-1).

For host range studies the plants species listed in Table 6-2 were mechanically inoculated. Plants were weekly inspected for symptom expression along 8 weeks, and then RT-PCR tested for the pepper viroid using the primer pair AP-FW1/RE2. In addition, for each plant species the RT-PCR product of one plant was sequenced. Potato plants (*Solanum tuberosum*) were grown for another 8 weeks at 18-20 °C for

Table 6-1. Primers used for RT-PCR amplification of *Pepper chat fruit viroid* (PCFVd)

Primer	Nucleotide sequence	Positions ^a
Pospi1-FW ^b	5'-GGG ATC CCC GGG GAA AC-3'	85-101
Pospi1-RE ^b	5'-AGC TTC AGT TGT (T/A)TC CAC CGG GT-3'	277-255
AP-FW1	5'-ACC CTT CCT TTC TTC GGG TTT CC-3'	178-200
AP-RE2	5'-CAG CGG GGA TTA CTC CTG TCA-3'	164-144
PCFVd-FW1 ^c	5'-ggt cta gAC CCT TCC TTT CTT CGG GTT TCC-3'	178-200
PCFVd-RE1	5'-GAA AAC CCT GTT TCA GCG GGG AT-3'	177-155

^a see Figure 6-2

^b specific for RT-PCR amplification of all pospiviroids except *Columnnea latent viroid* (Verhoeven et al., 2004); the first four nucleotides at the 5' terminus of Pospi1-RE do not match the PCFVd sequence

^c lower case characters refer to non-PCFVd nucleotides incorporated for cloning purposes

tuber production.

To compare the symptoms of the pepper viroid with PSTVd in pepper, plants of cv. Yolo Wonder were inoculated with the pepper viroid and two isolates of PSTVd, i.e. 'Howell' and '20011470' (Verhoeven et al., 2004). Prior to inoculation, the relative viroid concentration of the inocula was estimated by r-PAGE (Roenhorst et al., 2000; Schumacher et al., 1986) at dilutions 1:5, 1:10 and 1:50, with the plants being then inoculated with comparable viroid amounts. After 8 weeks, plants were tested for viroid accumulation by RT-PCR using the primer pair AP-FW1/RE2 (Table 6-1), and the temperature was decreased to 20-23 °C during night and day, respectively. Symptoms were recorded weekly for 14 weeks.

Transmission through seed

To examine transmission through seed, the mechanically inoculated plants of pepper cv. Yolo Wonder from the experiment to fulfill Koch's postulates were grown at 20-23 °C to induce fruit setting, and 72 seeds from three fruits were collected and stored at ca. 18 °C. After two weeks the seeds without any further treatment were sown in potting soil at 25 °C, and from two weeks after emergence plants were grown for 10 weeks at 20-23 °C, and then tested in groups of six plants by RT-PCR with the primer pair AP-FW1/RE2 (Table 6-1). Groups that reacted positively were subsequently tested individually, and finally, RT-PCR products of three plants were sequenced.

Nucleic acid extraction, RT-PCR amplification and PAGE

For molecular studies, nucleic acids were extracted from 1 g of young leaves with a Homex grinder (BioReba, supplied by Sanbio, Uden, NL) using an extraction bag containing 5 ml of extraction buffer (0.02 M PBS plus 0.05% Tween, 2% PVP and 0.2% ovalbumine). The RNA was purified with the PureScript kit from Gentra (Biozym, Landgraaf, NL) according to the manufacturer's instructions. The final RNA pellets were resuspended in 40 µl of the buffer included in the RT-PCR kit (see below).

For the initial RT-PCR amplifications one set of primers (AP-FW1/RE2) was developed in addition to the Pospi1-RE/FW pair (Verhoeven et al., 2004) (Table 6-1). RT-PCR was performed in a 25 µl reaction volume (containing 1 µl of the RNA preparation and 1.0 µM of each primer), using the SuperScript™ III One-Step RT-PCR System with Platinum® *Taq* DNA polymerase (Invitrogen, Breda, NL) according to manufacturer's instructions. RT-PCR amplification of the full-length PCFVd was performed with SuperScript™ II and *Pwo* DNA polymerase (Roche Applied Science), using the primer pair PCFVd-FW1/RE1 (Table 6-1). The resulting products were separated by PAGE in non-denaturing 5% gels, and the DNA of the expected full-length was eluted, digested with *Xba*I and cloned into pUC18 opened with *Sma*I and *Xba*I.

The circular forms of PCFVd and the two viroid standards, *Citrus exocortis viroid* (CEVd) and *Hop stunt viroid* (HSVd), were purified by double PAGE (Flores et al., 1985). Their relative mobilities were examined by PAGE in 5% gels containing 0.225 M Tris-borate-EDTA and 8 M urea (Sänger et al., 1977), and silver staining.

Sequence analysis, prediction of secondary RNA structure, and phylogenetic studies

The nucleotide sequences of PCFVd partial-length uncloned RT-PCR products was determined by BlaseClear in Leiden, NL, and that of full-length cloned RT-PCR products by an ABI 3100 Genetic Analyzer (Applied Biosystems) in Valencia, Spain. Multiple sequence alignments were performed using BLASTn (Altschul et al., 1990) and Clustal W (Thompson et al., 1994). The predicted viroid RNA secondary structure was obtained using Mfold, version 3.2 (Mathews et al., 1999; Zuker, 2003) and RnaViz, version 2 (De Rijk et al., 2003). Finally, phylogenetic analyses were conducted using MEGA, version 4 (Tamura et al., 2007). The nucleotide sequence of PCFVd has been deposited in the NCBI GenBank (Accession No FJ409044).

Results

Description of the disease in pepper

Near the end of the growing season in September 2006, first symptoms of a new disease were recorded in a glasshouse with four hectares of pepper cv. Jaguar. Fruit size of the affected plants was reduced by as much as half of the normal size (Figure 6-1A). Furthermore, plant growth was slightly reduced, and the young leaves of the infected plants were slightly smaller and paler than those of healthy plants. The affected plants were mainly located next to each other, in a few rows near the rear end of the glasshouse. At the end of May 2007, the disease reappeared in plants of the next cultivation of pepper cv. Easy, again in a few adjacent rows at the same location in the glasshouse. By taking hygienic measures the disease was contained to a few rows for several months. Only at the end of the growing season did a few plants at other locations in the glasshouse show symptoms. Stringent hygienic measures were taken during crop rotation at the end of 2007.

Fulfilling Koch's postulates

No viruses were detected by electron microscopy and mechanical inoculation to herbaceous test plants (data not shown); however, using RT-PCR with primers Posp1-RE/FW for the detection of eight of nine known pospiviroids, a PCR product of the expected half viroid-genome size was obtained. Sequencing and alignment of the PCR-product presumed the presence of a viroid species of the genus *Pospiviroid*, although only limited identities with known pospiviroids were found (see below).

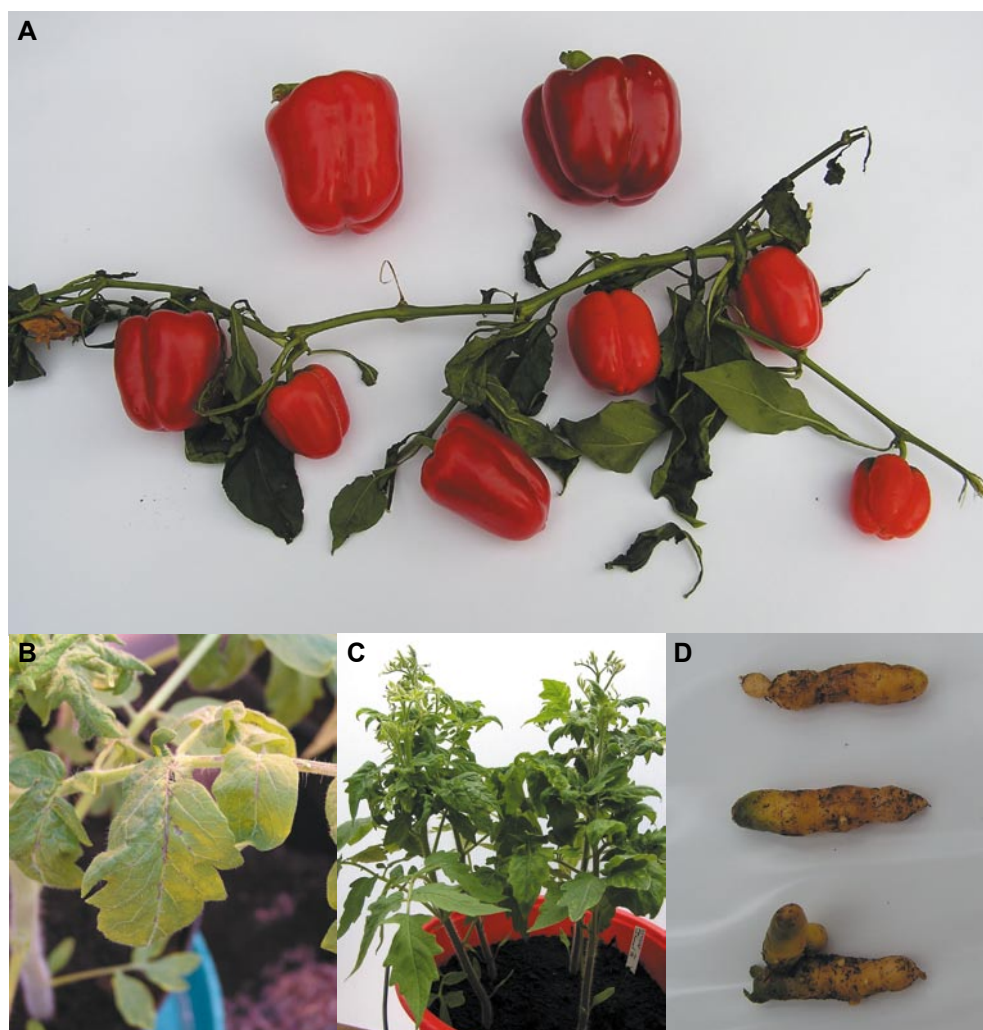


Figure 6-1. (A) Fruits of pepper cv Jaguar infected with *Pepper chat fruit viroid* (PCFVd) (bottom) compared with fruits from a healthy plant (top). (B) Necrosis along the veins and on the petioles of tomato cv Money-maker three weeks after mechanical inoculation with PCFVd. (C) Stunting of tomato cv Money-maker five weeks after mechanical inoculation with PCFVd. (D) Small, elongated and distorted tubers of potato plants cv Nicola inoculated with PCFVd.

The presumed viroid RNA was separated from other RNAs and DNAs by r-PAGE and then eluted and mechanically inoculated to plants of pepper cv. Yolo Wonder. Examination of the pepper plants 6 weeks after inoculation by RT-PCR, using the primer pair AP-FW1/RE2, showed the presence of the pepper viroid-like RNA in all inoculated plants. Furthermore, comparison of inoculated and non-inoculated plants after 12 weeks revealed a severe fruit size reduction in the inoculated plants. Hence, the viroid-like RNA isolated from pepper was indeed a viroid, which was

Table 6-2. Plant reaction after mechanical inoculation of *Pepper chat fruit viroid*.

Plant species	Symptoms
<i>Brugmansia suaveolens</i> cv. Geel	ns ^a
<i>Capsicum annuum</i> cv. Yolo Wonder	ns
<i>Lycianthes rantonnetii</i> , syn. <i>Solanum rantonnetii</i>	ns
<i>Solanum lycopersicum</i> cv. Moneymaker	d, gr, ld, vn
<i>Solanum jasminoides</i>	ns
<i>Solanum melongena</i> cv. Black Beauty	ns
<i>Solanum tuberosum</i> cv. Nicola	nl, td, tgr

^a d = discoloration, gr = growth reduction, ld = leaf distortion, nl = necrotic lesions, ns = no symptoms (symptomless infection), td = tuber distortion, tgr = tuber growth reduction, vn = veinal necrosis

then tentatively called *Pepper chat fruit viroid* (PCFVd) after its most conspicuous symptoms.

Experimental host range

In PCFVd-inoculated tomato (*Solanum lycopersicum*) cv. Moneymaker, young leaves showed necrotic spots and streaks along the veins and on the petioles (Figure 6-1B) 2 to 3 weeks after inoculation. Even if these symptoms did not always occur, they appeared characteristic for this viroid because, under similar environmental conditions, we have never observed them in tomato plants infected by other viroids. New leaves that developed two to three weeks after inoculation were small and a little distorted; their color was light-green, and later bronze or purple (Figure 6-1C). Furthermore, the growth of the tomato plants was severely reduced. The type of discoloration and stunting was similar to the severe type of symptoms incited by other tomato-infecting pospiviroids. In potato cv. Nicola, occasionally, PCFVd also evoked necrotic lesions on the leaves and petioles two to three weeks after inoculation. No further leaf symptoms were observed on potato. The tubers, however, appeared misshapen: they only measured one fourth to half of the normal size, were elongated, and produced lateral extensions along the main tubers (Figure 6-1D).

No symptoms were recorded for the other inoculated plant species during 8 weeks (Table 6-2). Nevertheless, they were PCFVd-infected as shown by RT-PCR using the primer pair AP-FW1/RE2. Direct sequencing of the PCR product of one plant of each inoculated species revealed that all sequences were identical to the original PCFVd sequence from pepper, showing that no genome mutant had become dominant in the experimental hosts.

Symptoms of PCFVd and PSTVd on pepper

Six weeks after inoculation first flowers appeared on the mock-inoculated plants and fruit setting started, while fruit setting started 2 weeks later on the PSTVd-

Table 6-3. Symptomatology of *Pepper chat fruit viroid* (PCFVd) and *Potato spindle tuber viroid* (PSTVd) on mechanically inoculated plants of pepper cv. Yolo Wonder.

Inoculum ^a	Plant height (cm)			Number of fruits per plant		Maximum fruit size (cm)	
	6 wpi ^b	10 wpi	14 wpi	10 wpi	14 wpi	10 wpi	14 wpi
PCFVd (3259237)	19.2 (± 3.6) ^c	38.4 (± 8.3)	52.4 (± 11.1)	1.4 (0-4)	3.6 (0-6)	2.5 (± 0.7)	3.5 (± 1.0)
PSTVd (Howell)	23.2 (± 6.4)	42.2 (± 4.9)	51.8 (± 8.6)	3.0 (0-8)	5.8 (3-11)	4.0 (± 0.0)	4.4 (± 1.3)
PSTVd (20011470)	26.6 (± 1.5)	41.6 (± 2.3)	46.6 (± 5.9)	3.4 (2-5)	6.0 (4-9)	3.4 (± 0.5)	4.4 (± 0.6)
Mock- inoculated ^d	23.2 (± 2.9)	31.2 (± 5.5)	35.0 (± 5.1)	5.4 (3-10)	5.6 (3-10)	5.4 (± 0.9)	6.6 (± 0.9)

^a the specific isolate is indicated between paranthesis

^b weeks post inoculation

^c numbers are mean values per five plants, with the standard deviation (in plant height and maximum fruit size) and the variation (in number of fruits per plant) indicated between parenthesis

^d a plausible explanation for the reduced height of the mock-inoculate plants is provided in the main text

and PCFVd-inoculated plants. Ten weeks after inoculation, the mock-inoculated plants were both smaller and more chlorotic than the inoculated ones due to the energy and nutrients needed for fruit setting, which not only started earlier at the mock-inoculated plants but also was more successful (Table 6-3). Furthermore, fruit number was reduced by PCFVd, and maximum fruit size was reduced by both PCFVd and the two PSTVd isolates, although the effects were more pronounced in the case of PCFVd. Nevertheless, the symptoms of PSTVd on pepper were more severe than those reported before (Lebas et al., 2005). Eight weeks after inoculation, PCFVd and PSTVd infections were confirmed by RT-PCR in all inoculated plants.

PCFVd is transmitted through pepper seed

Fifty-nine seedlings were raised from seventy-two seeds from PCFVd-infected pepper fruits. Eleven seedlings appeared infected by PCFVd after testing by RT-PCR using the primer pair AP-FW1/RE2. This is an infection rate of approximately 19%, assuming that the non-emergence of 13 seeds is not correlated to viroid infection.

We ignore whether PCFVd is located inside and/or outside the seeds. Sequencing of the RT-PCR products from three infected pepper seedlings demonstrated that the predominant nucleotide sequence of the viroid had not changed during seed transmission.

Molecular characterization and classification of PCFVd

The primary structure of the viroid genome was determined by direct sequencing of the partially overlapping RT-PCR products obtained with the primer pairs Posp1-RE/FW and AP-FW1/RE2 (Table 6-1). PCFVd consists of 348 nt: 106 C (30.5%), 100 G (28.7%), 76 U (21.8%), and 66 A (19.0%), which makes a C+G

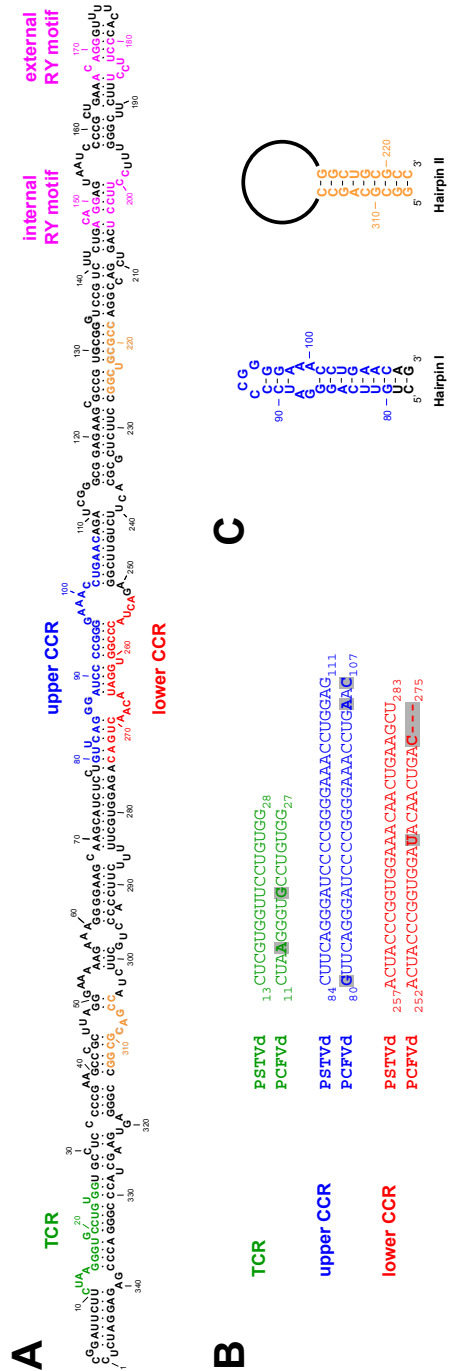


Figure 6-2. (A) Primary and proposed secondary structure of minimum free energy of *Pepper chat fruit viroid* (PCFVd). Nucleotides forming the terminal conserved region (TCR), upper and lower central conserved region (CCR), hairpin I and II, and internal and external RY motifs are highlighted in green, blue, red, orange and purple letters respectively. (B) Comparison between the TCR and the upper and lower CCR of PCFVd and *Potato spindle tuber viroid* (PSTVd), the type species of the genus *Pospiviroid*. Nucleotides conserved in pospirovirids (Flores et al., 1997), but not in PCFVd, are denoted in bold characters with a grey background. (C) Schematic representation of hairpin I and II that PCFVd and PSTVd can potentially form.

content of 59.2%. The computer-predicted secondary structure of minimum free energy is a rod-like conformation (Figure 6-2A) with 69.5% of the nucleotides paired (64.5% G:C, 29.7% A:U, and 5.8% G:U pairs). Accordingly with its size, examination by denaturing PAGE showed that PCFVd migrated with a mobility intermediate between that of CEVd (371 nt) and HSVd (297 nt) (Figure 6-3). The primary structure of PCFVd was confirmed by cloning the full-length RT-PCR products obtained with the primer pair PCFVd-FW1/RE1 (Table 6-1). Analysis of the inserts of seven recombinant plasmids revealed the same sequence.

The primary structure of PCFVd shows the presence of a CCR, which is the main criterion to classify a new viroid as a member of the family *Pospiviroidae*. Furthermore, the type of CCR and the presence of a TCR or a TCH are the main criteria for genus classification within this family (Flores et al., 1997; Flores et al., 2005b). The CCR of PCFVd closely resembles that of members from the genus *Pospiviroid*. Nevertheless, three and two nucleotide differences are observed in the upper and lower CCR strands, respectively, with the latter having a 3' terminus three nucleotide shorter (Figure 6-2B). The classification of PCFVd as a member of the genus *Pospiviroid* is further supported by the absence of a TCH and the presence of a TCR, in which two nucleotide differences are also found (Figure 6-2B). On the whole, PCFVd best matches the molecular characteristics of the genus *Pospiviroid*.

Further evidence for classifying PCFVd within the genus *Pospiviroid* was obtained from the conservation of other structural and sequence motifs. Like all members of the family *Pospiviroidae*, PCFVd can form one or two thermodynamically stable hairpins (Figure 6-2C). The structure of hairpin I resembles that of all genera of the family *Pospiviroidae* (Flores et al., 1997; Gas et al., 2007) including the apical palindromic tetraloop, the adjacent 3-bp stem, and the long 9-bp stem; however, it is most similar to that of the genus *Pospiviroid* and, interestingly, the three differences observed between the upper CCR strand of PCFVd and PSTVd (the type species of this genus) do not affect the stability of the stem of hairpin I because two are co-variations and the third transforms a G:U wobble into a G:C canonical base-pair. The lower strand of the rod-like secondary structure can alternatively form a stable hairpin (II) with a GC-rich stem of 9 bp resembling the hairpin II formed in PSTVd during thermal denaturation (Riesner et al., 1979; Loss et al., 1991); again, the sequence differences between PCFVd and PSTVd do not affect the stability of the stem of hairpin II because they are co-variations. In the terminal right domain of the rod-like secondary structure PCFVd also shows an internal and an external RY motif that has been proposed to mediate viroid systemic transport (Maniataki et al., 2003). Both, or at least one of these motifs, are present in all members of the genus *Pospiviroid* (Gozmanova et al., 2003).

A key criterion for viroid species demarcation is based on comparisons of the nucleotide sequence identity of the complete genomes (Flores et al., 2005b). For being considered a new species, a viroid must show less than 90% nucleotide

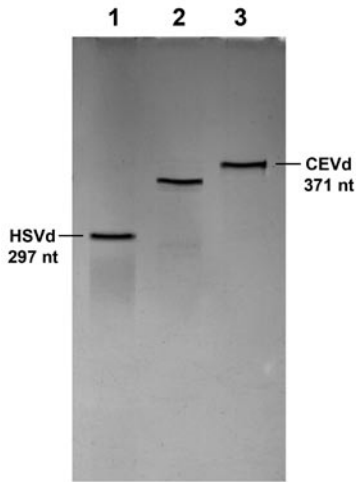


Figure 6-3. Mobilities of the purified circular forms of *Hop stunt viroid* (HSVd) (297 nt), *Pepper chat fruit viroid* (PCFVd) and *Citrus exocortis viroid* (CEVd) (371 nt) (lanes 1 to 3, respectively) after PAGE in 5% gels containing 0.225 M Tris-borate-EDTA and 8 M urea, and silver staining.

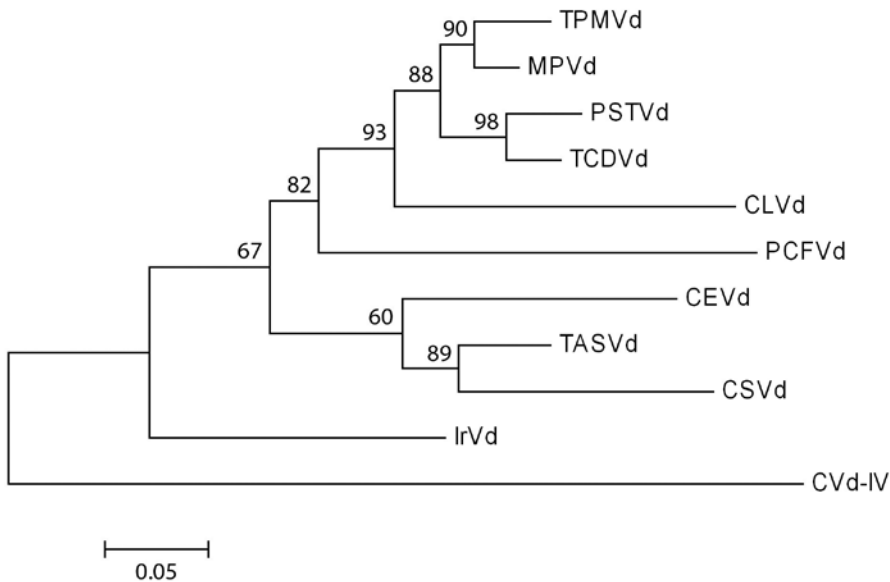


Figure 6-4. Evolutionary relationships of *Pepper chat fruit viroid* (PCFVd) and all current pospiviroids: *Tomato planto macho viroid* (TPMVd) (K00817), *Mexican papita viroid* (MPVd) (L78454), *Potato spindle tuber viroid* (PSTVd) (V01465), *Tomato chlorotic dwarf viroid* (TCDVd) (AF162131), *Columnnea latent viroid* (CLVd) (X15663), *Citrus exocortis viroid* (CEVd) (M34917), *Tomato apical stunt viroid* (TASVd) (NC001553) *Chrysanthemum stunt viroid* (CSVd) (X16407) and *Iresine viroid 1* (IrVd-1 (NC003613). *Citrus viroid IV* (Cvd-IV) (X14638) was added as an outgroup. The phylogenetic reconstruction was performed with the Neighbor-Joining method (Saitou & Nei, 1987). The fractions (%) of replicate trees in which the groups clustered together in the bootstrap test (5000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree, which were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are expressed in base substitutions per site. Phylogenetic analyses were conducted with MEGA4 (Tamura et al., 2007).

identity with the sequences from all known viroid species. Using BLASTn (Altschul et al., 1990), PCFVd shows the highest nucleotide sequence identities with members of the genus *Pospiviroid*; however, identities are less than 66%. As a consequence, the primary structure of PCFVd differs so much from all other viroids that creating a new species is justified on the basis of its molecular properties. Sequence differences are mainly located in the terminal left, pathogenic, and variable domains of the rod-like secondary structure (Keese & Symons, 1985), while the terminal right domain shows sequence identities even up to 100% with some isolates of *Columnnea latent viroid* (CLVd), *Mexican papita viroid* (MPVd), *Tomato apical stunt viroid* (TASVd) and *Tomato planta macho viroid* (TPMVd).

Phylogenetic reconstructions also support the classification of PCFVd as a new species of the genus *Pospiviroid*

Several phylogenetic analyses were performed including PCFVd, all current members of the genus *Pospiviroid*, *Citrus viroid* IV (CVd-IV, recently renamed *Citrus bark cracking viroid*, CBCVd, genus *Cocaviroid*), *Grapevine yellow speckle viroid* 1 (GYSVd-1, genus *Apscaviroid*) and HSVd (genus *Hostuviroid*). Figure 6-4 shows a phylogenetic tree based on the Neighbor-joining method (Saitou & Nei, 1987), in which PCFVd groups with the current members of the genus *Pospiviroid* while CVd-IV appears as an outgroup. Similar results were obtained when including GYSVd-1 or HSVd instead of CVd-IV (data not shown). Therefore, the phylogenetic analyses support the classification of PCFVd as a member of the genus *Pospiviroid*. Furthermore, the branch length of PCFVd in the phylogenetic trees is also consistent with considering it as new viroid species.

Discussion

Our present results show that a new pepper disease is caused by a viroid, PCFVd, which can be discriminated from other viroids by its biological and molecular properties. PCFVd is transmitted by both mechanical inoculation and pepper seeds, which is in line with previous observations with other pospiviroids infecting solanaceous plants (Singh et al., 1988; Singh & Dilworth, 2009), and, it can experimentally infect several solanaceous plants wherein, occasionally, it causes a new type of necrotic symptoms on potato and tomato leaves. The molecular properties of PCFVd also differ from viroids that have been reported so far, with which it only shows up to 66% sequence identity. However, the CCR and TCR of PCFVd mostly resemble those of the genus *Pospiviroid*, while a TCH is lacking as in other species of this genus. Furthermore, the presence and structure of hairpins I and II, and of internal and external RY motifs, also favor classification of PCFVd within the genus *Pospiviroid*, as well as the phylogenetic analyses showing that

PCFVd differs from current members of the genus *Pospiviroid* at the species but not at the genus level.

The origin of the PCFVd infection in pepper cv. Jaguar in 2006 is unclear. Although PCFVd is seed-borne, two reasons make unlikely that infected pepper seeds were the source of infection. First, the disease was noticed only at the end of the growing season whereas, in the case of seed transmission, symptoms would have been expected earlier. Secondly, the disease was only reported in a single crop of pepper cv. Jaguar, whereas more infections would have been expected considering that 40 hectares of pepper cv Jaguar were grown in 2006 and our estimate of a seed-transmission rate of nearly 20%. The infection of pepper cv. Easy by PCFVd in 2007 most probably originated from the infection in the previous crop because the glasshouse had not been cleaned and disinfected thoroughly during crop rotation.

The observation of the first symptoms in the crop of pepper cv. Jaguar only late in the growing season of 2006 suggests another host plant, probably from outside the glasshouse, as the source of infection. In addition to pepper cv. Jaguar, other plants in the glasshouse included: a group of 25 pepper plants of different varieties, a few ornamental plants of *Bougainvillea* sp. and *Dipladenia* sp., grown in the rear end of the glasshouse, and some weeds. Testing the 25 pepper plants in groups of five before their removal at the end of the growing season detected PCFVd in two groups (data not shown). Nevertheless, it is unlikely that these plants had acted as sources of infection because the presence of PCFVd in only part of them and their lack of symptoms rather suggest that they became infected later than the plants of pepper cv. Jaguar. Furthermore, testing all ornamental plants as well as some weeds of *Calystegia sepium*, *Equisetum* sp. and a fern did not reveal any PCFVd infection (data not shown). Because our study also shows that PCFVd can infect various solanaceous species, and pepper was the only species of this family present in the glasshouse, we presume that solanaceous plants from outside the glasshouse may have acted as the primary sources of infection in analogy with the situation for CEVd, CLVd and PSTVd infections of tomato (Verhoeven et al., 2004 and 2010).

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Chapter 7

General discussion

New diagnostic methods identifying new pospiviroids and hosts

During the last decade several pospiviroid infections have been identified in pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) crops in various parts of the world (e.g. Elliot et al., 2001; Lebas, 2005; Ling & Bledsoe, 2009; Ling et al., 2009; Mumford et al., 2004; Nixon et al., 2009; Steyer et al., 2009; Verhoeven et al., 2004, 2006a, 2007a, 2009b and 2010). Improved methods not only enabled a more sensitive detection but also more specific identification of pospiviroids. In the last twenty years of the 20th century pospiviroid detection was often based on either return-polyacrylamide gelelectrophoresis (rPAGE) or on hybridization assays. However, both methods did not discriminate between all viroid species and therefore could not be used for unequivocal identification. Especially, for *Potato spindle tuber viroid* (PSTVd) this was unsatisfactory because of its quarantine status in many countries. Not knowing the exact identity casted doubts on official measures to be enforced if PSTVd indeed would be present.

The introduction of reverse transcription-polymerase chain reaction (RT-PCR) for viroid detection (e.g. Önelge, 1996; Shamloul et al., 1997) provided a more sensitive tool for detection and enabled definite identification by the sequencing of complete genome-length PCR products. Using RT-PCR the occurrence of *Citrus exocortis viroid* (CEVd) and PSTVd was determined in tomato crops in the Netherlands (Chapter 2). In addition, PSTVd and *Tomato chlorotic dwarf viroid* (TCDVd) were identified in samples of tomato from New Zealand and the USA, respectively. The identification of this last viroid using primers developed for specific detection and identification of PSTVd (Shamloul et al., 1997) showed the need of sequencing for identification. The available primer sets, however, failed to detect and identify the viroid(s) from six tomato crops, previously detected by rPAGE. Therefore, two sets of degenerated primers were developed to react with all known pospiviroids. RT-PCR with primer pair Pospi1-RE/FW amplified half of the viroid genome of all pospiviroids except *Columnea latent viroid* (CLVd), and subsequent sequencing of the amplicons enabled preliminary identification. Primer pair Vid-RE/FW enabled amplification of the complete genome of CLVd as well as that of PSTVd and TCDVd. RT-PCRs with this primer pair and subsequent sequencing of the PCR product identified all remaining tomato viroid isolates as CLVd (Chapter 2). In addition, the relevance of primer pair Pospi1-RE/FW was demonstrated by the preliminary identification of a new pospiviroid species *Pepper chat fruit viroid* (PCFVd) by sequencing the half viroid-genome-length amplicon from diseased pepper plants (Chapter 6) and of *Tomato apical stunt viroid* (TASVd) in a tomato sample from Tunisia (Verhoeven et al., 2006a).

The identification of the various pospiviroids in the seed-propagated crop

of tomato raised questions on the origin of the infections. It was generally assumed that PSTVd infections in tomato originated from infested seed lots, since seed transmission had been reported frequently in both potato (*Solanum tuberosum*) and tomato (Fernow et al., 1969; Kryczynski et al., 1988; Singh, 1970). In addition, results from studies on seed transmission of PSTVd were generally translated to other pospiviroids, even without experimental proof (Singh et al., 1999; Singh et al., 2003). With regard to the pospiviroid infections in tomato in the Netherlands, however, no indications had been obtained that either seeds or plants for planting had been the sources of infection. Since CLVd only had been reported from ornamental plants in the families *Gesneriaceae* and *Solanaceae*, surveys were initiated in plants belonging to these families to trace alternative viroid sources.

As a result of these surveys many new viroid/host plant combinations were detected by using primer pairs Pospi1-RE/FW and Vid-RE/FW (Verhoeven et al., 2006b, 2007b, 2008a, b and c). At the same time Bostan et al. (2004), Nie et al. (2005) and Singh et al. (2006) also identified several new viroid/host combinations by using a primer pair nearly identical to Pospi1-RE/FW. Currently, ca. 75% of all known pospiviroid/host combinations have been revealed by using semi-universal primer pairs (Table 1). Remarkably, CLVd - the viroid identified most frequently in tomato in the Netherlands in the 1990s and also identified elsewhere, ever since (Nixon et al., 2009; Steyer et al., 2009; JThJ Verhoeven, unpublished results) - was not encountered in any of the ornamental plants during surveys in the Netherlands since 2006. In 2009, however, the viroid was discovered in single plants of three *Gloxinia* spp. in a botanical garden in Denmark (Table 1; SL Nielsen, personal communication). Despite this recent finding principle ornamental hosts of CLVd have not been found, yet.

The surveys in the Netherlands, however, also revealed large numbers of PSTVd infections in *Brugmansia* spp. and *S. jasminoides* (Chapter 3). Other new viroid/ornamental host combinations were identified only once or twice, i.e. *Chrysanthemum stunt viroid* (CSVd) in *Petunia hybrida* and *S. jasminoides*, *Iresine viroid 1* (IrVd-1) in *Portulaca* sp. and TASVd in *Lycianthes rantonnetii* and *Streptosolen jamsonii* (Verhoeven et al., 1998, 2006b, and unpublished results). Other combinations were encountered more frequently, e.g. PSTVd in *L. rantonnetii* (Di Serio, 2007; JThJ Verhoeven, unpublished results) and TASVd in both *Cestrum* sp. and *S. jasminoides* (Verhoeven et al., 2008a, c, and unpublished results). So, many new viroid/ornamental host combinations were found with different degrees of infection.

In the Netherlands, no new PSTVd infections were found in both *Brugmansia* spp. and *Solanum jasminoides* after growing season 2006/2007. This was the result of both the eradication measures and the introduction of the plant-passport system in June 2007 (De Hoop et al., 2008). Infections by TASVd, however, were still found repeatedly in both *Cestrum* sp. and *S. jasminoides*. For *Cestrum* sp. this

Table 7-1. Ornamental (o) and wild (w) host plants of members of the genus *Pospiviroid*.

<i>Pospiviroid</i>	Host plants	References
<i>Chrysanthemum stunt viroid</i>	<i>Ageratum</i> sp. (o)	Henkel & Snger, 1995
	<i>Argyranthemum frutescens</i> (o)	Menzel & Maiss, 2000
	<i>Chrysanthemum x morifolium</i> (o)	Haseloff & Symons, 1981
	<i>Dahlia</i> sp. (o)	Nakashima et al., 2007
	<i>Pericallis x hybrida</i> (o)	ETM Meekes, personal communication
	<i>Petunia</i> sp. (o)	Verhoeven et al., 1998
	<i>Solanum jasminoides</i> (o)	Verhoeven et al., 2006b
	<i>Verbena</i> sp. (o)	Bostan et al., 2004*
	<i>Vinca major</i> (o)	Bostan et al., 2004* ;Nie et al., 2005
<i>Citrus exocortis viroid</i>	<i>Glandularia pulchella</i> (o/w)	Singh et al., 2006*
	<i>Impatiens</i> sp. (o)	Bostan et al., 2004*; Nie et al., 2005
	<i>Solanum jasminoides</i> (o)	Verhoeven et al., 2008c
	<i>Verbena</i> sp. (o)	Singh et al., 2006*; Verhoeven et al., 2008a
<i>Columnnea latent viroid</i>	<i>Brunfelsia undulata</i> (o)	Spieker, 1996a
	<i>Columnnea erythrophye</i> (o)	Hammond et al., 1989
	<i>Gloxinia gymnostoma</i> (o/w)	SL Nielsen, personal communication
	<i>G. nematanthodes</i> (o/w)	SL Nielsen, personal communication
	<i>G. purpurascens</i> (o/w)	SL Nielsen, personal communication
	<i>Nematanthus wettsteinii</i> (o)	Singh et al., 1992a
	<i>Solanum stramonifolium</i> (w)	NCBI GenBank No. AM698095
<i>Iresine viroid 1</i>	<i>Alternanthera sessilis</i> (o/w)	Singh et al., 2006*
	<i>Celosia plumosa</i> (o)	JThJ Verhoeven, unpublished results
	<i>Iresine herbstii</i> (o)	Spieker, 1996b
	<i>Portulaca</i> sp. (o)	JThJ Verhoeven, unpublished results*
	<i>Verbena</i> sp. (o)	Bostan et al., 2004
	<i>Vinca major</i> (o)	Bostan et al., 2004
	<i>Brugmansia x candida</i> (o)	Verhoeven et al., 2010
<i>Potato spindle tuber viroid</i>	<i>Brugmansia x flava</i> (o)	Verhoeven et al., 2010
	<i>Brugmansia sanguinea</i> (o)	Verhoeven et al., 2010
	<i>Brugmansia suaveolens</i> (o)	Verhoeven et al., 2008a; Verhoeven et al., 2010
	<i>Calibrachoa</i> sp. (o)	JThJ Verhoeven, unpublished results
	<i>Datura</i> sp. (o)	Verhoeven et al., 2010
	<i>Lycianthes rantonnetii</i> (o)	Di Serio, 2007
	<i>Petunia</i> sp. (o)	Mertelik et al., 2009
	<i>Solanum jasminoides</i> (o)	Verhoeven et al., 2008a; Verhoeven et al., 2010
	<i>Streptosolen jamesonii</i> (o)	Verhoeven et al., 2008b

* identification based on circa half of the sequence of the viroid genome

Table 7-1 (continued). Ornamental (o) and wild (w) host plants of members of the genus *Pospiviroid*

<i>Pospiviroid</i>	Host plants	References
<i>Tomato apical stunt viroid</i>	<i>Cestrum</i> sp. (o)	Verhoeven et al., 2008a
	<i>Lycianthes rantonnetii</i> (o)	JThJ Verhoeven, unpublished results
	<i>Solanum jasminoides</i> (o)	Verhoeven et al., 2008c
	<i>Solanum pseudocapsicum</i> (o)	Spieker et al., 1996
	<i>Streptosolen jamesonii</i> (o)	JThJ Verhoeven, unpublished results
<i>Tomato chlorotic dwarf viroid</i>	<i>Brugmansia sanguinea</i> (o)	Verhoeven et al., 2010
	<i>Petunia hybrida</i> (o)	Verhoeven et al., 2007b
	<i>Pittosporum tobira</i> (o)	ABH Elleuch, personal communication
	<i>Verbena</i> sp. (o)	Singh et al., 2006*
	<i>Vinca minor</i> (o)	Singh & Dilworth, 2009

* identification based on circa half of the sequence of the viroid genome

is not surprising because growers were not forced to remove the infected plants. For *S. jasminoides* most plants had been eradicated because of infections by PSTVd. The finding of TASVd infections in this crop can be explained by the fact that the assay used for screening the samples from the survey in *S. jasminoides* did not detect all pospiviroids but only *Mexican papita viroid* (MPVd), PSTVd and TCDVd (Boonham et al., 2004; JThJ Verhoeven, unpublished results). Starting vegetative propagation from PSTVd-free plants or newly imported - PSTVd but not pospiviroid tested - plants may, therefore, explain the partial replacement of PSTVd by TASVd in *S. jasminoides*.

In addition to their use for surveying pospiviroids, the combination of primer pairs Pospi1-RE/FW and Vid-RE/FW offers a great potential for indexing (candidate) mother plants of vegetatively propagated ornamentals. The efficient transmission of viroids by vegetative propagation requires solid guarantee of viroid absence. Evidence was obtained that indeed all ten characterized pospiviroids will be detected by using RT-PCRs with these two primer sets. Moreover, the robustness of the RT-PCR assays was demonstrated by the outcome of a ring test as part of the ERA-net EUPHRESKO project on Detection and Epidemiology of Pospiviroids (DEP). Furthermore, the detection of the previously unknown PCFVd in pepper plants showed that the combination of the primer sets may detect pospiviroids not characterized at the time of testing.

The high sensitivity of the new (real-time) RT-PCR methods is a major advantage in comparison with the less sensitive methods used for pospiviroid detection and identification, previously. This advantage may be a major drawback at the same time, however. Inherent to the use of very sensitive methods is the risk of false-positive reactions because of cross-contamination (Borst et al., 2004). Prevention of false-positive results is a major challenge for successful application of

the new RT-PCR techniques. Both adequate numbers of negative controls, as well as verification of positive results are required to exclude false positives. In infected tomato crops that show viroid symptoms, the presence of symptoms will support positive RT-PCR results. However, symptoms cannot be used for confirmation when testing symptomless seeds or ornamental plants. Especially in these cases, alignment of viroid sequences from the diagnostic samples with those from the positive controls and other recently analysed, positive samples should be performed. In the case of high sequence identities, tests should be repeated starting with the RNA isolation from the original samples. In this way successful application of PCR assays can be guaranteed.

Vegetatively propagated, solanaceous crops as sources of pospiviroid inoculum for tomato

The finding of many pospiviroid infections in ornamental crops poses the risk that these crops act as inoculum sources for tomato. This risk was substantiated by successful mechanical transmission of pospiviroids from ornamentals to potato and tomato (Chapter 3-5). Moreover, the predominant pospiviroid genotypes appeared very stable upon transmission, which could explain the phylogenetic relationships among PSTVd isolates from tomato with isolates from vegetatively propagated, solanaceous host species. Phylogenetic analyses revealed the existence of four clusters of variants recovered from *Brugmansia* spp., *Physalis peruviana*, potato and *S. jasminoides*. Two factors may explain the clustering of PSTVd variants from *P. peruviana* in a single cluster despite the fact that this host is partially propagated by seed, i.e. its perennial nature under semi-tropical conditions and the absence of symptoms. The PSTVd variants from the merely seed-propagated tomato did not form a separate cluster but were distributed among several other clusters (Figure 3-3). This indicates that the vegetatively propagated crops had acted as sources of infection for the PSTVd outbreaks in tomato. Navarro et al. (2009) also associated an outbreak of PSTVd in tomato with infected plants of *S. jasminoides* in Italy. So, for PSTVd conclusive evidence has been obtained that vegetatively propagated, solanaceous crops act as sources of inoculum for tomato. These results concur with those of Sano et al. (2001), who also found clustering of *Hop stunt viroid* (HSVd) isolates according to host plants and geographic distribution and deduced grapevine as the origin of the viroid in Japanese hop.

The phylogenetic analyses of PSTVd variants revealed four clusters of sequences, each associated with a vegetatively propagated plant species (Figure 3-3). These plant species should be considered *sensu lato*. The cluster of sequences originating from potato also includes wild potato species like *Solanum commersonii* (Van Wezenbeek et al., 1982). This can be explained by the fact that both wild and

cultivated potatoes have been used for breeding, thus enabling transmission of PSTVd either mechanically or by seed and pollen. Also the cluster of sequences from *S. jasminoides* contains sequences from other vegetatively propagated plant species, such as *Lycianthes rantonnetii* (Di Serio, 2007), *Streptosolen jamesonii* (Verhoeven et al., 2008b) and *Calibrachoa* cv. Sun and Blue (JThJ Verhoeven, unpublished results). Considering the high number of infected *S. jasminoides* plants, this species probably also has been the main source of inoculum for transfer by chance to the other ornamentals and tomato.

In the cluster of *P. peruviana*, this plant species is the only (partially) vegetatively propagated crop, so far. The majority of infections by PSTVd variants from this cluster have been identified in Australia and New Zealand (Chapter 3). This suggests that PSTVd was introduced there with *P. peruviana* when it was carried as a vegetable from South America via South Africa a few centuries ago. Although this crop is far less frequently grown in Western Europe than *S. jasminoides*, the PSTVd infections in tomato in Belgium and the UK (Mumford et al., 2004; Verhoeven et al., 2007a) seem to relate to this plant species (Chapter 3). Therefore, it could also be considered that the PSTVd genotypes from *P. peruviana* might have infected other plant species that in turn acted as sources of infection for seed-propagated crops like tomato. In this way the plants of pepino (*Solanum muricatum*) probably acted as an intermediate source of inoculum for the PSTVd outbreak in two tomato crops in the Netherlands in 1988 (Puchta et al., 1990). However, no further sources of PSTVd inoculum for tomato have been found in this cluster, so far.

Similar to PSTVd, also no relations to seed lots and plants for planting have been found for the outbreaks of CEVd and CLVd in tomato in the Netherlands. The same holds true for the majority of pospiviroid outbreaks in tomato elsewhere. To reveal potential relations, phylogenetic analyses were performed with the available genotypes of CLVd, TASVd and TCDVd, for which the original plant species was known (Figure 7-1). For all three pospiviroids substantially less sequences are available than for PSTVd, which makes it more speculative to draw conclusions from the phylogenetic analyses. Nevertheless, some interesting clustering can be observed.

For CLVd the sequences from tomato isolates originating from France, Portugal and the UK group in a single cluster (Figure 7-1a). All isolates from tomato in France and the UK originate from the same variety, i.e. cv. Santa (Nixon et al., 2009; Steyer et al., 2009), the tomato variety in Portugal was not reported. Together with their high mutual sequence identities this indicates that these infections probably originated from the same contaminated seed lot. The viroid still may have been introduced from infected ornamental plants, but then during seed propagation of the variety. Furthermore, the tree shows similar clustering of sequences as shown in Figure 2-5, except for two sequences from Thailand, which group in a separate cluster. One of these sequences is from tomato, the other from *Solanum stramonifolium*, a

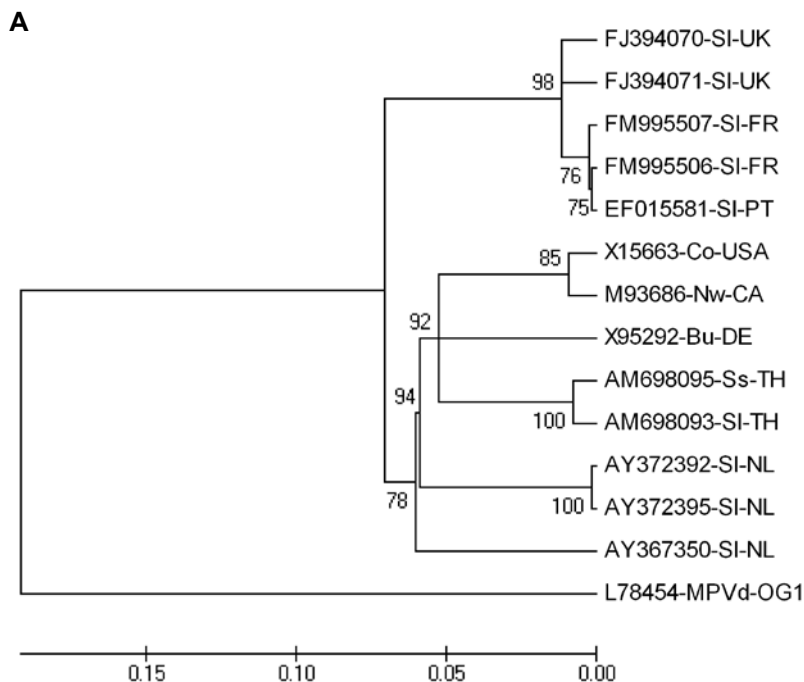
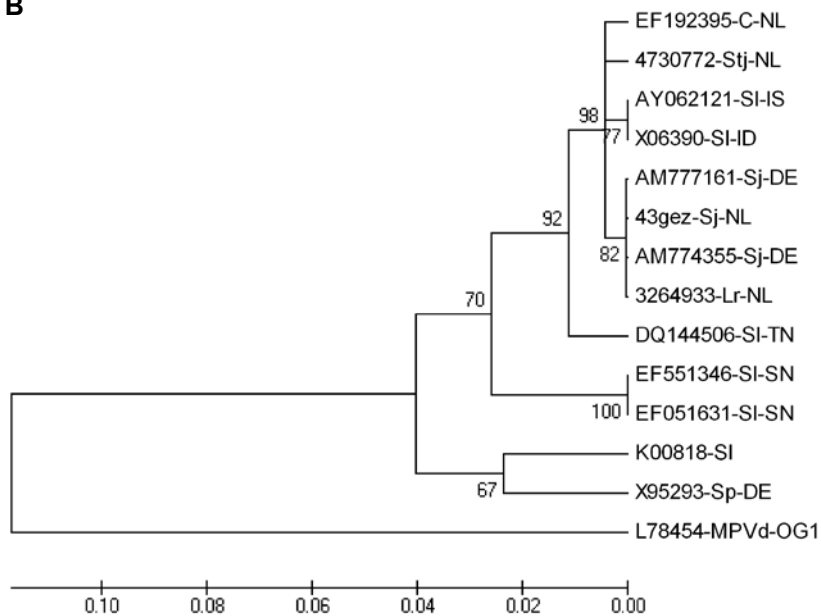


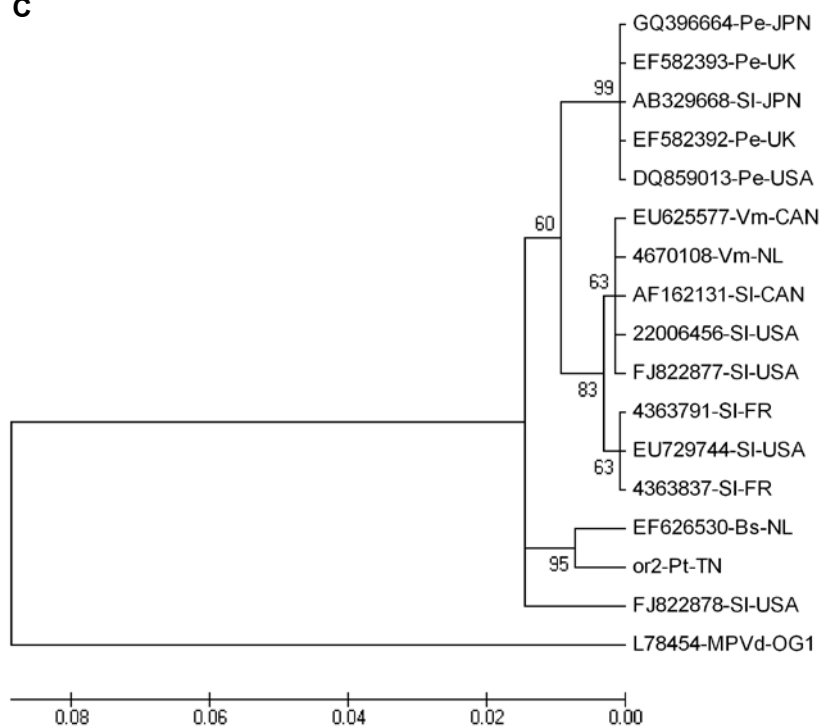
Figure 7-1. Evolutionary relationships of taxa from a) *Columnnea latent viroid* (CLVd), b) *Tomato apical stunt viroid* (TASVd) and c) *Tomato chlorotic dwarf viroid* (TCDVd).

The evolutionary histories were inferred using the Minimum Evolution method (Rzhetsky & Nei, 1992). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown next to the branches (Felsenstein, 1985). The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. To overcome complete gap deletion, sequences were slightly adapted. The ME trees were searched using the Close-Neighbor-Interchange (CNI) algorithm at a search level of 1 (Nei & Kumar, 2000). The Neighbor-joining algorithm was used to generate the initial trees (Saitou & Nei, 1987). Other phylogenetic methods produced similar trees. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). *Mexican papita viroid* (NCBI GenBank No. L78454) was used as an outgroup for all analyses. Acronyms were used for the countries, in which isolates originated. (A) Evolutionary relationships of 14 CLVd genotypes from *Brunsfelsia undulata* (Bu), *Columnnea erythrophye* (Co), *Nematanthus wettsteinii* (Nw), *Solanum stramonifolium* (Ss) and tomato (SI). (B) Evolutionary relationships of 14 TASVd genotypes from *Cestrum* sp. (C), *Lycianthes rantonnetii* (Lr), *Solanum jasminoides* (Sj), *Solanum pseudocapsicum* (Sp), *Streptosolen jamesonii* (Stj) and tomato (SI). (C) Evolutionary relationships of 17 TCDVd genotypes from *Brugmansia sanguinea* cv. Oro Verde (Bs), *Petunia* sp. (Pe), *Pittosporum tobira* (Pt), tomato (SI) and *Vinca minor* (Vm). (22006456-SI-USA is a corrected sequence previously submitted to the NCBI-GenBank as AY372399.)

B



C



wild solanaceous plant species. Since there is no publication reporting on these sequences, we can only speculate on the role of the latter plant species as inoculum source. For the other CLVd infections in tomato no relations to ornamentals or wild plants have been found, yet.

Concerning TASVd, two tomato isolates reported in Israel and Indonesia group with sequences from *Cestrum* sp., *L. rantonnetii*, *S. jasminoides* and *S. jamesonii*, suggesting that infected plants of the latter species might have been the inoculum source (Figure 7-1b). However, since plants of *Cestrum* sp. and *S. jasminoides* have been found infected frequently, and *L. rantonnetii* and *S. jamesonii* only once, the latter two probably were also infected from *Cestrum* sp. and/or *S. jasminoides*. This hypothesis is substantiated by the fact that these four crops are often propagated and grown together. Furthermore, four TASVd tomato isolates, two from Senegal, one probably originally from Ivory Coast (K00818) and one from Tunisia, are separated according to the country of origin. Only the sequence from Ivory Coast shows some relationship with a German sequence from *Solanum pseudocapsicum*, an ornamental propagated both by cuttings and seed. For the other infections in tomato no potential sources of inoculum could be identified, yet.

For TCDVd the phylogenetic analysis shows three clusters and one separate sequence (Figure 7-1c). The first cluster contains all sequences from *Petunia* sp. from various countries and one sequence from tomato in Japan. This indicates that the infection in tomato in Japan might originate from *Petunia* plants. This is substantiated by the fact that the TCDVd sequence from tomato was identical to the sequence of the viroid intercepted in *Petunia* plants from Japan (NCBI GenBank Nr. GQ396664). The second cluster represents the majority of TCDVd sequences from tomato. Since these sequences group with two isolates from the vegetatively propagated *Vinca minor* (Singh & Dilworth, 2009; JThJ Verhoeven, unpublished results), this indicates that *V. minor* may have been a source of inoculum for several outbreaks in tomato. The third cluster contains the TCDVd sequences from the ornamentals *Brugmansia sanguinea* cv. Oro Verde and *Pittosporum tobira*. Finally, the tomato isolate FJ822878 from Arizona, USA, is separated from all other sequences, and as a consequence no source of inoculum can be indicated.

In summary, phylogenetic analyses show that similar to PSTVd, ornamental crops may have been the source of inoculum for the outbreaks in tomato of TASVd and TCDVd. For CLVd, there are indications that several infections in tomato crops were related to the use of contaminated seed, although inoculum sources could not be traced, so far. Nevertheless, these observations show that ornamental plants can be potential sources of pospiviroid inoculum and therefore, call for vigilance to prevent infections in potato and tomato crops.

Introduction of pospiviroids in potato and tomato

Viroids detected in crops were assumed to originate from wild host plants (Diener, 1996). Transmission to cultivated plants is supposed to occur either by chance transfer or by breeding activities with germplasm of wild plants. Despite many efforts, however, PSTVd has not been found in plants grown in the wild so far, and also infections of wild plants by other pospiviroids are rare (Table 7-1). This study showed that viroid infections in tomato may also originate from cultivated plants, in particular solanaceous ornamentals that are propagated vegetatively and do not show symptoms. In contrast, wild plants are mainly propagated by true seed. Potentially, these seeds may be infected by pospiviroids, but transmission rates will be substantially lower than for vegetative propagation. Furthermore, viroid-infected wild plants mainly spread locally, whereas infected ornamental plants have been spread worldwide because of extensive international trade. Therefore, contacts between infected ornamentals and potato or tomato are more likely than for infected wild plants. As a consequence, transfer of pospiviroids to the latter crops by chance may occur more frequently from ornamentals than from wild plants. Hence, it should be concluded that these infected plants without symptoms in commercially grown crops are more important in the epidemiology of pospiviroids than wild plants.

In the case of potato, which is mainly propagated vegetatively, a single or a few introductions of PSTVd may have been sufficient for extended infections in this crop. Infections in breeding lines and varieties persist during the regular ways of propagation. By trading infected seed potatoes the viroid was spread over potato growing areas worldwide. For example, PSTVd infections in potato in China and South Korea are assumed to have been introduced from North America via infected seed potatoes of the variety 'Irish Cobbler' (Singh et al., 1993). Further spread in the same crop was the result of mechanical transmission during crop handling and probably also aphid transmission.

For tomato, solely propagated by true seed, the epidemiology of pospiviroids is quite different. The absence of vegetative propagation prevents the most efficient way of persistence in a crop. New seedlings replace mature plants at least once a year, and therefore, a viroid infection will not persist for long periods of time unless seeds of infected plants are being used for propagation, continuously. The latter is unlikely, considering the severity of the symptoms in tomato, which presumably results in discarding of infected plants before seed harvesting. Generally, this implies that viroid outbreaks in tomato crops result from new introductions from external sources.

New pospiviroid outbreaks occur less frequently in potato than tomato. Over the last 20 years, only PSTVd has been identified in potato, whereas seven different pospiviroids have been found in tomato. In potato the recent PSTVd infections were

Table 7-2. PSTVd infections in potato between 1988 and 2009, and their presumed source of infection.

Year	Country	Source of infection	Reference
1989	?	potato breeding material ^a	Verhoeven et al., 2010
1990	?	potato breeding material ^a	Verhoeven et al., 2010
1990	PL	potato breeding material ^a	Verhoeven et al., 2010
1991	CN	seed potatoes	Singh et al., 1991; Singh et al., 1993
1993	RU	potato breeding material ^a	Roenhorst & Verhoeven, 1995; Verhoeven et al., 2010
1994	?	potato breeding material ^a	Verhoeven et al., 2010
1994	CU	potato breeding material ^a	Roenhorst & Verhoeven, 1995; Verhoeven et al., 2010
1997	DE	potato breeding material ^a	Roenhorst & Verhoeven, 1998; Verhoeven et al., 2010
1997	CR	seed potatoes	Badilla et al., 1999
1998	CR	seed potatoes	Badilla et al., 1999
1998	BY	seed potatoes	Blotskaya & Berlinchik, 1998
1998	DE	potato breeding material	NPPO of Germany, 2000
1998	IS	potato breeding material ^a	Roenhorst & Verhoeven, 2000; Verhoeven et al., 2010
2001	FR	potato breeding material	NPPO of France, 2002
2002	RU	seed potatoes ^b	Kastalyeva et al., 2007; Verhoeven et al., 2010
2007	RU	seed potatoes	Kastalyeva et al., 2007; Owens et al., 2009
2009	MT	unknown ^c	B de Hoop, personal communication

^a interceptions during post-entry quarantine testing in the Netherlands

^b sample received from T Kastalyeva

^c report concerns two 'suspect' cases of PSTVd infection in potato from 2008; however, cross-contamination during testing could not be excluded. Subsequent surveys in 2009 did not show any infections

derived from infected seed potatoes or breeding material. New PSTVd outbreaks are unknown (Table 7-2), possibly with one exception being a dubious report from Malta (B de Hoop, personal communication). The recent infections in tomato (Table 7-3) may be explained by the fact that this crop is more susceptible to PSTVd. In addition, tomato is generally grown at higher temperatures than potato, which favours viroid infections (Chapter 5). Furthermore, there is more intensive crop handling by hands and scissors in tomato, which both have been shown effective for the introduction of PSTVd. Finally, ornamental crops will generally be grown more close to tomato than potato crops, thereby increasing the chance of viroid transmission. All these arguments presumably will also hold true for the other pospiviroids because of their highly identical biological properties.

For the reported findings of PSTVd in tomato since the first outbreak of PSTVd in tomato in the Netherlands in 1988, nearly all PSTVd infections appear to originate from either *P. peruviana* or *S. jasminoides* (Table 7-3). Two infections might originate from potato, and no infections could be related to *Brugmansia* spp. The relatively high number of PSTVd infections in tomato related to *S. jasminoides* may be explained by the fact that this ornamental is by far the most frequently

Table 7-3. PSTVd infections in tomato between 1988 and 2009, and their presumed source of infection based on sequence identities between PSTVd genotypes from tomato and other solanaceous crops.

Year	Country	Source of infection	Reference
1988	NL	<i>Physalis peruviana</i>	Puchta et al., 1990
1988	NL	<i>P. peruviana</i>	Puchta et al., 1990
1994	NL	potato ^a	Verhoeven et al., 2004
2000	NL	<i>Solanum jasminoides</i>	Verhoeven et al., 2010
2001	NZ	<i>P. peruviana</i>	Verhoeven et al., 2004; Elliott et al., 2001
2001	NL	<i>S. jasminoides</i>	Verhoeven et al., 2004 and 2010
2002	AUS	<i>P. peruviana</i>	Mackie et al., 2002
2003	AUS	<i>S. jasminoides</i>	Hailstones et al., 2003
2003	DE	<i>S. jasminoides</i>	Verhoeven et al., 2010
2003	UK	<i>P. peruviana</i>	Mumford et al., 2004
2005	CAN	<i>P. peruviana</i>	Verhoeven et al., 2010
2006	BE	<i>P. peruviana</i>	Verhoeven et al., 2007a
2006	USA	<i>S. jasminoides</i>	Verhoeven et al., 2010
2006	USA	potato	Verhoeven et al., 2010
2008 ^b	IS	tomato seed / <i>S. jasminoides</i>	R Gottsberger, personal communication
2008 ^b	NL/DE	tomato seed / <i>S. jasminoides</i>	NPPO of Austria. 2008
2009	IT	<i>S. jasminoides</i>	Navarro et al., 2009

^a cross-contamination during propagation of the isolate cannot be excluded

^b infections only detected in seed lots; positive results were only obtained in one laboratory and could not be confirmed by other laboratories

grown ornamental of these host plants worldwide. In the Netherlands infected lots of *S. jasminoides* contained substantially more plants than those of *Brugmansia* spp. (Chapter 3). Furthermore, the PSTVd genotypes from *S. jasminoides* have also been identified in other vegetatively propagated ornamentals (Di Serio, 2007; Verhoeven et al., 2008b; JThJ Verhoeven, unpublished results) that could have been additional sources of inoculum. Finally, the fact that none of the PSTVd infections in tomato appeared related to *Brugmansia* spp. might have been due to the less efficient viroid transmission from this species (Chapter 5).

The relative increase of PSTVd infections in tomato since 2000 most likely resulted from an increase in the total number of inoculum sources. Several solanaceous host plants have become more popular during the last decade. As a consequence, the numerous symptomless infections in these crops may have contributed to the increase of PSTVd infections in tomato. However, the recognition of viroid symptoms should not be neglected either. Initially, the first PSTVd infections

in tomato in the Netherlands (1988) and New Zealand (1999/2000) had not been recognized as such. The testing for viroids only started circa 6 and 16 months after first symptoms had been observed, respectively. This suggests that viroid infections may have remained at some more locations. Finally, also the detection and identifications tools have improved in the last decade. This together with the large number of inoculum sources and increased knowledge on viroids might have contributed the relatively high number of pospiviroid reports in tomato.

Quarantine status for pospiviroids?

Currently, PSTVd and CSVd are the only two pospiviroids with an explicit quarantine status in the European Union (EU). The quarantine status of PSTVd applies to all host plant species, whereas the status of CSVd is restricted to planting material of chrysanthemum (*Chrysanthemum x morifolium*). The potential of causing severe diseases in potato, tomato and chrysanthemum, respectively, is the reason for specific regulation of these pospiviroids. In addition, PSTVd was assumed to be absent in the EU and the quarantine status intended to prevent its introduction via seed potatoes and potato germplasm, which were considered to be the main sources of inoculum. In contrast, CSVd was known to occur in the EU and therefore, regulation aimed to prevent the introduction into plants for planting of its main host chrysanthemum. The recent findings of other pospiviroids and 'new' host plants, however, ask for evaluation of the regulatory status of pospiviroids.

At least for the pospiviroids found in tomato a comparison with PSTVd seems logical. First, similar symptoms were observed in all infected tomato crops, irrespective of the viroid concerned, i.e. CEVd, CLVd, PSTVd, TASVd or TCDVd. Also for MPVd and *Tomato planta macho viroid* (TPMVd) similar symptoms have been reported (Galindo et al., 1982; Ling & Bledsoe, 2009). Furthermore, after mechanical inoculation onto potato cv. Nicola and tomato cv. Moneymaker all five pospiviroids from tomato included in our studies evoked more or less similar symptoms (Chapter 2; JThJ Verhoeven, unpublished results). Secondly, for all these viroids similar ways of spread have been observed in the infected tomato crops. This is not surprising because similar ways of transmission are known for all pospiviroids. Finally, all pospiviroids have at least multiple host plants, in addition to potato and tomato.

Besides the seven tomato infecting pospiviroids, PCFVd should also be evaluated because of its similarity in experimental host range, symptomatology and ways of transmission. Evaluation of IrVd-1 can be ignored because only symptomless infections have been recorded in some ornamental plants, whereas potato and tomato could not be infected experimentally (Spieker, 1996b; JThJ Verhoeven, unpublished results). Finally, CSVd should be evaluated separately since potato has not been reported as an (experimental) host and no infections have been reported in

commercially grown tomato.

Exploring the situation for the seven tomato-infecting pospiviroids shows that the highest economical impact has been recorded for potato and tomato. Since the first two infections by PSTVd in tomato in the Netherlands in 1988 (Puchta et al., 1990), fifteen new PSTVd outbreaks have been reported in tomato crops (Table 7-3). Although there may have been some increase of PSTVd infections in tomato crops during the last decade, the total number of new outbreaks is still very limited, i.e. one or two reported cases per year, worldwide. In contrast to the low number of outbreaks in tomato, the number of ornamentals plants infected by various pospiviroids, especially PSTVd, was found to be high. Moreover, strong indications have been obtained that PSTVd infections in ornamentals date back many years (Chapter 3) and, as a consequence, the identification of PSTVd in various ornamental plants is new as a finding but not as a fact. So, symptomless sources of inoculum occurred in the past and will also have acted as such, and only the number of plants acting as sources of inoculum may have increased, since. The number of outbreaks by PSTVd in tomato, though, has been small. This also holds true for other pospiviroids. So, these data would not justify severe regulation.

Presently, PSTVd infections in potato are rare in most countries, and many countries have even declared freedom of PSTVd and/or reported the eradication of the viroid e.g. De Boer & De Haan (2005), EPPO (n.d.) and Sun et al. (2004). All recent reports on PSTVd infections - except for a dubious report from Malta - can be associated with the use of infected seed potatoes or infected germplasm for breeding (Table 7-2). Hence, introducing this type of potato material forms by far the highest risk of introducing PSTVd. As a consequence, for regulation measures are needed for this type of plant material but can be limited for other types.

Not taking any measures to control pospiviroid outbreaks, however, might lead to severe damage in pepper, potato and tomato, at least occasionally. The highest risks for serious damage can be expected after infection of basic planting material of these crops. Therefore, specific measures could be taken to prevent such infections as well as to eradicate them in the case of an outbreak. Prevention measures could be taken for all propagation stages, but emphasis should be given to the top of the production columns. Obtaining and maintaining viroid-free stock material, testing and certification are key elements for successful prevention. In addition, hygienic measures contribute to the prevention of new viroid introductions. Of course, additional measures would be needed in all propagation stages in case pospiviroids have been accidentally introduced. For potato, measures should include destruction of infected lots and temporary areal absence of pospiviroid host plants including eradication of volunteer plants and weeds. Infected tomato lots will also have to be destroyed. However, temporary areal absence of pospiviroid hosts could be limited to a short period when adequate measures are taken to thoroughly clean and disinfect the particular greenhouses. Obligatory measures for tomato and

pepper growers producing fruits for consumption could be omitted or restricted to some elementary measures during the replacement of the old plants at the end of the growing season. In contrast, compulsory measures might be needed for growers of ware potatoes because pospiviroid control is more complicated due to volunteer plants here. In addition, - temporary - control measures could be considered for mother plants of other hosts for the production both of cuttings (mainly ornamentals) and seeds (e.g. *P. peruviana*), when high infection rates occur in these crops. So, future control of pospiviroids would not require defined measures for all growers of pospiviroid host plants but only for producers of planting material with the highest risks to act as sources of inoculum. By balancing its measures, future governmental quarantine policy can lead agriculture to economically safe and durable production.

If all pospiviroids except CSVd and IrVd-1 should be nominated for the same quarantine status, the question remains which status. A quarantine status is determined after 'pest risk analysis' (PRA) by various specialists, a.o. economists and virologists. In PRA the likelihood of successful invasion by plant pests is estimated. This includes an evaluation of the chances for introduction, establishment and further spread. In addition, the economic, social and environmental impact on successful invasions should be estimated. To make the correct estimations, sufficient data on pest identity, geographic distribution, host range, transmission, economic and environmental losses etc. are needed. However, many relevant data were still lacking when a reassessment of the quarantine statuses of pospiviroids was first proposed (Verhoeven et al., 2004). Many issues have been addressed since; however, four topics still need further attention.

The identification of a recently found, deviating pospiviroid in tomato from Mexico is the first issue to be addressed because the outcome may have consequences for pospiviroid taxonomy. Currently, the main species demarcation criterion is less than 90% nucleotide sequence identity of the complete viroid genome (Flores et al., 2005b). In addition, the criterion of different biological characteristics is used, i.e. mainly host range and symptomatology. However, this latter criterion is seldom applied because the sequence identity criterion usually clearly identifies the viroid. Biological characteristics have been used, however, to support the identification of some pospiviroid isolates from tomato as CLVd because their sequence identity to previously characterized variants of CLVd was less than 90% (Chapter 2). In addition, biological characteristics were used to discriminate MPVd and TPMVd, whose sequences showed more than 90% identity (Martínez-Soriano et al., 1996). Also the newly discovered Mexican pospiviroid cannot be identified on the basis of sequence identity alone, because its 93 and 92% identity with MPVd and TPMVd, respectively, would allow identification as either of these two species. So, biological data are needed to decide on the identity of this new variant. The results of the biological studies, however, may have consequences for the current classification of MPVd and TPMVd as two separate viroid species because the reported biological

characteristics are not in line (Galindo et al., 1982; Martínez-Soriano et al., 1996). In turn, the outcome may affect PRA in case individual pospiviroid species will be addressed.

The second issue to be further studied is the transmission of pospiviroids by seeds because of contradictory results. In 1999 Singh et al. had not detected TCDVd in both seeds and seedlings from infected tomato plants. Koenraadt et al. (2009), however, detected the viroid in all seeds from TCDVd-infected tomato plants but did not detect the viroid in 4000 seedlings. In contrast Singh & Dilworth (2009), using another batch of TCDVd-infected tomato seeds, detected the viroid in circa 90% of the seeds and also in 80% of the seedlings. For CLVd, there is circumstantial evidence that the viroid was transmitted via tomato seeds from a contaminated lot (Nixon et al., 2009), but experiments in the Netherlands, using 100 seeds from CLVd infected tomato plants, had not yielded any infected seedling (AW Werkman, personal communication). These contradictory results might be related with the location of the viroid in or at the tomato seeds. Disinfection of tomato seed using sodium hypochlorite did not prevent seed transmission of TCDVd (Singh & Dilworth, 2009). Similar results were obtained for PSTVd transmission via tomato seeds by S Winter (personal communication) when using sodium hypochlorite, hydrochloric acid or pectinase as disinfectants. These results suggest that TCDVd and PSTVd are not only located outside the seeds but also inside. However, then the questions remain which inner parts of the seeds become infected, and does this affect the transmissibility? In addition, the role of viroid variants and different tomato varieties should be taken into consideration.

Thirdly, there is a need for sensitive pospiviroid detection methods in bulked samples of seeds because controlling pospiviroids also requires adequate tools for detection and identification. To allow testing of adequate samples of large seed lots bulking is necessary, which requires very sensitive assays. In addition to real-time RT-PCR for MPVd, PSTVd and TCDVd (Boonham et al., 2004; JThJ Verhoeven unpublished results) new real-time RT-PCR assays have been developed for CEVd, CLVd and TASVd in the ERA-net EUPHRESKO-DEP project, recently (WA Monger, personal communication). Promising preliminary results using these assays for PSTVd, TCDVd (Koenraadt et al., 2009) and CLVd (A Fox, personal communication) in tomato seeds have been obtained. Maximum numbers of seeds in bulked samples varied from 100 – 1000. Since viroid concentrations between individual seeds vary considerably, the maximum number of seeds that may be bulked in a single sample still needs to be determined. A newly developed real-time RT-PCR for the detection of six pospiviroids allows detection of most pospiviroids in a single assay but is less sensitive than the specific real-time RT-PCRs (WA Monger, personal communication) and therefore, may lack sensitivity for testing bulked samples of many seeds.

The last aspect for further study concerns the potential transmission of pospiviroids by insects. Transmission by aphids has been demonstrated for PSTVd

in the case of mixed infections with *Potato leafroll virus* (PLRV) (Querci et al., 1997; Syller et al., 1997). The prerequisite that inoculum sources of PSTVd should be coinfecting with PLRV strongly reduces the role of aphids in the epidemiology of pospiviroids. Their role might increase, however, if other pospiviroids could be transmitted in a similar way and if transmission could also occur when inoculum sources are coinfecting by other viruses. In addition, bumble bees – normally used as pollinators - have been reported to transmit TASVd and TCDVd within tomato crops (Antignus et al., 2007; Matsuura et al., 2010). However, studies in the EUPHRESKO-DEP programme using bumble bees for transmission of PSTVd from *Petunia* sp. to tomato did not reveal any viroid transmission via those potential vectors (M Virscek-Marn, personal communication). Furthermore, also honey bees and thrips (both *Frankliniella occidentalis* and *Thrips tabaci*) failed to transmit PSTVd from ornamentals to tomato (SL Nielsen & R Gottsberger, personal communications). The discrepancy between these findings and the results from Antignus et al. (2007) and Matsuura et al. (2010) might be explained by the fact that the latter experiments were performed within tomato crops. This might have enabled transmission by pollination and subsequent fertilization with contaminated pollen, which is unlikely in the case of viroid transmission between different plant species. A further elucidation of the principle of viroid transmission by bumble bees in tomato may help to assess the role of these and other insects in viroid transmission.

In summary this thesis describes the improvement of detection and identification of pospiviroids by RT-PCR. The design of two novel primer sets to detect all known pospiviroids in an array of potential hosts adds to sensitivity of the analysis and enables identification after sequencing of the amplicons. The primers also had a prominent role in the detection and characterization of the new pospiviroid: *Pepper chat fruit viroid*. Furthermore, a critical analysis of the epidemiology of pospiviroids claims an important role for ornamental plants as sources of inoculum for tomato (and potato), and ways of mechanical viroid transmissions from ornamentals to both vegetables have been demonstrated. The data presented in this thesis contribute to a better preparedness for new pospiviroid infections and provide relevant information for a (re)assessment of the quarantine statuses of tomato-infecting pospiviroids.

References

- Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ. 1990.** Basic local alignment search tool. *Journal of Molecular Biology* 215, 403-410.
- Ambros S, Hernández C, Desvignes J & Flores R. 1998.** Genomic structure of three phenotypically different isolates of peach latent mosaic viroid: Implications of the existence of constraints limiting the heterogeneity of viroid quasispecies. *Journal of Virology* 72, 7397-7406.
- Anonymous. 2008.** Survey results for the presence of *Potato spindle tuber viroid* in the European Union in 2007. Brussels, Belgium: European Commission, 5pp (restricted distribution).
- Antignus Y, Lachman O & Pearlsman M. 2007.** Spread of *Tomato apical stunt viroid* (TASVd) in greenhouse tomato crops is associated with seed transmission and bumble bee activity. *Plant Disease* 91, 47-50.
- Badilla R, Hammond R & Rivera C. 1999.** First report of potato spindle tuber viroid in Costa Rica. *Plant Disease* 83, 1072.
- Barbosa CJ, Pina JA, Pérez-Panadés J, Bernad L, Serra P, Navarro L & Duran-Vila N. 2005.** Mechanical transmission of citrus viroids. *Plant Disease* 89, 749-754.
- Baumstark T & Riesner D. 1995.** Only one of four possible secondary structures of the central conserved region of potato spindle tuber viroid is a substrate for processing in a potato nuclear extract. *Nucleic Acids Research* 23, 4246-4254.
- Baumstark T, Schröder ARW & Riesner D. 1997.** Viroid processing: switch from cleavage to ligation is driven by a change from tetraloop to a loop E conformation. *The EMBO Journal* 16, 599-610.
- Behjatnia SAA, Dry IB, Krake LR, Condé BD, Connelly MI, Randles JW & Rezaian MA. 1996.** New potato spindle tuber viroid and tomato leaf curl geminivirus strains from a wild *Solanum* sp. *Phytopathology* 86, 880-886.
- Blotskaya ZV & Berlinchik EE. 1998.** Occurrence and harmfulness of potato spindle tuber viroid (PSTV) in the republic of Belarus. *Archives of Phytopathology and Plant Protection* 31, 453-457.
- Bonfiglioli RG, Mc Fadden GI & Symons RH. 1994.** In situ hybridization localizes avocado sunblotch viroid on chloroplast thylakoid membranes and coconut cadang cadang viroid in the nucleus. *The Plant Journal* 6, 99-103.
- Boonham N, Gonzáles Pérez L, Mendez MS, Lilia Peralta E, Blockly A, Walsh K, Barker I & Mumford RA. 2004.** Development of a real-time RT-PCR assay for the detection of *Potato spindle tuber viroid*. *Journal of Virological Methods* 116, 139-146.
- Borst A, Box ATA & Fluit AC. 2004.** False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. *European Journal of Clinical Microbiology & Infectious Diseases* 23, 289-299.
- Bostan H, Nie X & Singh RP. 2004.** An RT-PCR primer pair for the detection of pospiviroid and its application in surveying ornamental plants for viroids. *Journal of Virological Methods* 166, 189-193.
- Branch AD & Robertson HD. 1984.** A replication cycle for viroids and other small infectious RNAs. *Science* 223, 450-455.

- Branch AD, Benenfeld BJ & Robertson HD. 1985.** Ultraviolet light-induced crosslinking reveals a unique region of local tertiary structure in potato spindle tuber viroid and HeLa 5S RNA. *Proceedings of the National Academy of Sciences of the United States of America* 82, 6590–6594.
- Brierley P & Smith FF. 1951.** Chrysanthemum stunt. Control measures effective against virus in florists' crops. *Florists' Review* 107, 27-30.
- Carbonell A, Martínez de Alba A-E, Flores R & Sago S. 2008.** Double-stranded RNA interferes in a sequence-specific manner with the infection of representative members of the two viroid families. *Virology* 371, 44-53.
- Chen W, Yang XC & Po T. 1987.** Pear rusty skin, viroid disease. In *Abstracts of the 7th International Virology Conference* (p 300) Edmonton, Canada.
- Chung BN & Pak HS. 2008.** Seed transmission of *Chrysanthemum stunt viroid* in *Chrysanthemum*. *The Plant Pathology Journal* 24, 31-35.
- Codoñer FM, Daròs JA, Solé RV & Elena SF. 2006.** The fittest versus the flattest: Experimental confirmation of the quasispecies effect with subviral pathogens. *PloS Pathogens* 2(12), e136. (doi:10.1371/journal.ppat.0020136).
- Daròs JA & Flores R. 2002.** A chloroplast protein binds a viroid RNA in vivo and facilitates its hammerhead-mediated self-cleavage. *The EMBO Journal* 21, 749-759.
- Daròs JA, Marcos JF, Hernández C & Flores R. 1994.** Replication of avocado sunblotch viroid: Evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. *Proceedings of the National Academy of Sciences of the United States of America* 91, 12813-12817.
- Daròs JA, Elena SF & Flores R. 2006.** Viroids: an Ariadne's thread into the RNA labyrinth. *EMBO reports* 7, 593–598.
- De Boer SH & De Haan TL. 2005.** Absence of Potato spindle tuber viroid within the Canadian potato industry. *Plant Disease* 89, 910.
- De Bokx JA & Piron PGM. 1981.** Transmission of potato spindle tuber viroid by aphids. *Netherlands Journal of Plant Pathology* 87, 31-34.
- De Hoop MB, Verhoeven JThJ & Roenhorst JW. 2008.** Phytosanitary measures in the European Union: a call for more dynamic risk management allowing more focus on real pest risks. Case study: *Potato spindle tuber viroid* (PSTVd) on ornamental *Solanaceae* in Europe. *EPPO Bulletin* 38, 510-515.
- De La Peña M, Navarro B & Flores R. 1999.** Mapping the molecular determinant of pathogenicity in a hammerhead viroid: A tetraloop within the in vivo branched RNA conformation. *Proceedings of the National Academy of Science of the United States of America* 96, 9960-9965.
- De la Peña M & Flores R. 2002.** Chrysanthemum chlorotic mottle viroid RNA: Dissection of the pathogenicity determinant and comparative fitness of symptomatic and non-symptomatic variants. *Journal of Molecular Biology* 321, 411-421.
- Delgado S, Martínez de Alba AE, Hernández C & Flores R. 2005.** A short double-stranded RNA motif of Peach latent mosaic viroid contains the initiation and self-cleavage sites of both polarity strands. *Journal of Virology* 79, 12934-12943.
- Denti MA, Boutla A, Tsagris M & Tabler M. 2004.** Short interfering RNAs specific for potato spindle tuber viroid are found in the cytoplasm but not in the nucleus. *The Plant Journal* 37, 762-796.
- De Rijk P, Wuyts J & De Wachter R. 2003.** RnaViz 2: an improved representation of RNA secondary structure. *Bioinformatics* 19, 299-300.
- Diener TO. 1971.** Potato spindle tuber "virus" IV Replicating, low molecular weight RNA. *Virology* 45, 411-428.

- Diener TO. 1981.** Are viroids escaped introns? Proceedings of the National Academy of Sciences of the United States of America 78, 5014-5015.
- Diener TO. 1986.** Viroid processing: A model involving the central conserved region and hairpin I. Proceedings of the National Academy of Sciences of the United States of America 83, 58-62.
- Diener TO. 1989.** Circular relics of precellular evolution? Proceedings of the National Academy of Sciences of the United States of America 86, 9370-9374.
- Diener TO. 1996.** Origin and evolution of viroids and viroid-like satellite RNAs. Virus Genes 11, 119-131.
- Diener TO. 1999.** Viroids and the nature of viroid diseases. Archives of Virology, Supplement 15, 203-220.
- Diener TO. 2001.** The viroid: Biological oddity or evolutionary fossil? Advances in Virus Research 57, 137-184.
- Diener TO & Raymer WB. 1967.** Potato spindle tuber virus: A plant virus with properties of a free nucleic acid. Science 158, 378-381.
- Diener TO & Raymer WB. 1969.** Potato spindle tuber virus: A plant virus with properties of a free nucleic acid. II Characterization and partial purification. Virology 37, 351-366.
- Diener TO & Raymer WB. 1971.** Potato spindle tuber 'virus'. CMI/AAB Descriptions of Plant Viruses No. 66. 4pp.
- Diener TO, Hammond RW, Black T & Katze MG. 1993.** Mechanism of viroid pathogenesis: Differential activation of interferon-induced, double-stranded RNA-activated Mr 68,000 protein kinase by viroid strains of varying pathogenicity. Biochimie 75, 533-538.
- Ding B & Itaya A. 2007.** Viroid: a useful model for studying the basic principles of infection and RNA biology. Molecular Plant-Microbe Interaction 20, 7-20.
- Ding B, Kwon MO, Hammond R & Owens R. 1997.** Cell-to-cell movement of potato spindle tuber viroid. The Plant Journal 12, 931-936.
- Di Serio F. 2007.** Identification and characterization of *Potato spindle tuber viroid* infecting *Solanum jasminoides* and *S. rantonnetii* in Italy. Journal of Plant Pathology 89, 297-300.
- Domingo E & Holland JJ. 1997.** RNA virus mutations and fitness for survival. Annual Review of Microbiology 51, 151-178.
- Eigen M & Biebricher CK. 1988.** Sequence space and quasispecies distribution. In: Domingo E, Holland JJ & Ahlquist P, eds. RNA Genetics Vol. 3. CRC Press, Boca Raton, Florida, USA. pp 211-245.
- Elena SF & Sanjuán R. 2007.** Virus evolution: Insights from an experimental approach. Annual Review of Ecology, Evolution and Systematics 38, 27-52.
- Elena SF, Dopazo J, Flores R, Diener TO & Moya A. 1991.** Phylogeny of viroid, viroidlike satellite RNAs, and the viroidlike domain of hepatitis d virus RNA. Proceedings of the National Academy of Sciences of the United States of America 88, 5631-5634.
- Elliott DR, Alexander BJR, Smales TE, Tang Z & Clover GRG. 2001.** First report of *Potato spindle tuber viroid* in tomato in New Zealand. Plant Disease 85, 1027.
- EPPO. nd.** Data sheet on quarantine pest, Potato spindle tuber viroid. Retrieved December 31, 2009, from http://www.eppo.org/QUARANTINE/virus/PSTVd/PSTVD0_ds.pdf.
- EU Council Directive 2000/29/EC. nd.** Retrieved December 6, 2009, from <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2000L0029:20090303:EN:PDF>.
- Fagoaga C & Duran-Vila N. 1996.** Naturally occurring variants of citrus exocortis viroid in vegetable crops. Plant Pathology 45, 45-53.
- Fels A, Hu K & Riesner D. 2001.** Transcription of potato spindle tuber viroid by RNA polymerase II starts predominantly at two specific sites. Nucleic Acids Research 29, 4589-4597.

- Felsenstein J. 1985.** Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783-791.
- Fernow KH, Peterson LC & Plaisted RL. 1970.** Spindle tuber virus in seeds and pollen of infected potato plants. *American Potato Journal* 41, 75-80.
- Flores R, Duran-Vila N, Pallás V & Semancik JS. 1985.** Detection of viroid and viroid-like RNAs from grapevine. *Journal of General Virology* 66, 2095-2102.
- Flores R, Di Serio F & Hernández C. 1997.** Viroids: the non-coding genomes. *Seminars in Virology* 8, 65-73.
- Flores R, Daròs JA & Hernández C. 2000a** *Avsunviroidae* family: viroids with hammerhead ribozymes. *Advances in Virus Research* 55, 271-323.
- Flores R, Randles JW, Bar-Joseph M & Diener TO. 2000b.** Viroids. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB, Estes MK, Lemon SM, Maniloff J, Mayo MA, McGeoch DJ, Pringle CR & Wickner RB (eds). *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*. Academic Press, San Diego, USA. pp 1009-1024.
- Flores R, Delgado S, Gas ME, Carbonell A, Molina D, Gago S & De la Peña M. 2004.** Viroids: the minimum non-coding RNAs with autonomous replication. *FEBS Letters* 567, 42-48.
- Flores R, Hernández C, Martínez de Alba AE, Daròs JA & Di Serio F. 2005a.** Viroids and viroid-host interactions. *Annual Review of Phytopathology* 43, 117-139.
- Flores R, Randles JW, Owens RA, Bar-Joseph M & Diener TO. 2005b.** Subviral agents: Viroids. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U & Ball LA (eds). *Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses*. Elsevier Academic Press, San Diego, USA. pp 1147-1161.
- Gago S, Elena SF, Flores R & Sanjuán R. 2009.** Extremely high variability of a hammerhead viroid. *Science* 323, 1308 (doi: 10.1126/science.1169202).
- Galindo J, Smith DR & Diener TO. 1982.** Etiology of planta macho, a viroid disease of tomato. *Phytopathology* 72, 49-54.
- Galindo J, Lopez M & Aguilar T. 1986.** Significance of *Myzus persicae* in the spread of tomato planta macho viroid. *Fitopatologia Brasileira* 11, 400-410.
- Gas M-E, Hernández C, Flores R & Daròs J-A. 2007.** Processing of nuclear viroids in vivo: an interplay between RNA conformations. *PLoS Pathogens* 3, 1813-1825.
- Gómez G & Pallás V. 2001.** Identification of an in vitro ribonucleoprotein complex between a viroid RNA and a phloem protein from cucumber plants. *Molecular Plant Microbe Interactions* 14, 910-913.
- Gómez G & Pallás V. 2004.** A long-distance translocatable phloem protein from cucumber forms a ribonucleoprotein complex in vivo with I RNA. *Journal of Virology* 78, 10104-10110.
- Gómez G, Torres H & Pallás V. 2005.** Identification of translocatable RNA-binding phloem proteins from melon, potential components of the long-distance RNA transport system. *The Plant Journal* 41, 107-116.
- Goss RW. 1926.** Transmission of Potato spindle-tuber disease by cutting-knives and seed piece contact. *Phytopathology* 16, 68-69.
- Gozmanova M, Denti MA, Minkov IN, Tsagris, M & Tabler M. 2003.** Characterization of the RNA motif responsible for the specific interaction of potato spindle tuber viroid RNA (PSTVd) and the tomato protein Virp1. *Nucleic Acids Research* 31, 5534-5543.
- Grasmick ME & Slack SA. 1985.** Symptom expression enhanced and low concentrations of potato spindle tuber viroid amplified in tomato with high light intensity and temperature. *Plant Disease* 69, 49-51.

- Gross HJ, Domdey H, Lossow C, Jank P, Raba M, Alberty H & Sanger HL. 1978.** Nucleotide sequence and secondary structure of potato spindle tuber viroid. *Nature* 273, 203-208.
- Haas B, Klanner A, Ramm K & Sanger HL. 1988.** The 7S RNA from tomato leaf tissue resembles a signal recognition particle RNA and exhibits a remarkable sequence complementarity to viroids. *The EMBO Journal* 7, 4063-4074.
- Hadidi A, Hansen AJ, Parish CL & Yang X. 1991.** Scar skin and dapple apple viroids are seed-borne and persist in infected apple trees. *Research in Virology* 142, 289-296.
- Hadidi A, Giunchedi L, Shamloul AM, Poggi-Pollini C & Amer MA. 1997.** Occurrence of peach latent viroid in stone fruits and its transmission with contaminated blades. *Plant Disease* 81, 154-158.
- Hailstones DL, Tesoriero LA, Terras MA & Dephoff C. 2003.** Detection and eradication of *Potato spindle tuber viroid* in tomatoes in commercial production in New South Wales, Australia. *Australasian Plant Pathology* 32, 317-318.
- Hammond RW. 1994.** Agrobacterium-mediated inoculation of PSTVd cDNAs onto tomato reveals the biological effect of apparently lethal mutations. *Virology* 201, 36-45.
- Hammond RW. 2003.** *Columnea* latent viroid. In: Hadidi A, Flores R, Randles JW & Semancik JS (eds). *Viroids*. CSIRO Publishing, Collingwood, Australia. pp 231-232.
- Hammond R, Smith DR & Diener TO. 1989.** Nucleotide sequence and proposed secondary structure of *Columnea* latent viroid: a natural mosaic of viroid sequences. *Nucleic Acids Research* 17, 10083-10094.
- Handley MK & Horst RK. 1988.** The effect of temperature and light on chrysanthemum stunt viroid infection of florist chrysanthemum. *Acta Horticulturae* 234, 89-97.
- Harders J, Lukacs N, Robert-Nicoud M, Jovin TM & Riesner D. 1989.** Imaging of viroids in nuclei from tomato leaf tissue by in situ hybridization and confocal laser scanning microscopy. *The EMBO Journal* 8, 3941-3949.
- Haseloff J & Symons RH. 1981.** Chrysanthemum stunt viroid – primary sequence and secondary structure. *Nucleic Acids Research* 9, 2741-2752.
- Henkel J & Sanger HL. 1995.** NCBI GenBank Accession Number Z68201.
- Hernandez C & Flores R. 1992.** Plus and minus RNAs of peach latent mosaic viroid self-cleave in vitro via hammerhead structures. *Proceedings of the National Academy of Sciences of the United States of America* 89, 3711-3715.
- Hiddinga HJ, Crum CJ, Hu J & Roth DA. 1988.** Viroid-induced phosphorylation of a host protein related to dsRNA-dependent protein kinase. *Science* 241, 451-453.
- Holland J, Spindler K, Horodyski F, Grabau E, Nichol S & Vande Pol S. 1982.** Rapid evolution of RNA genomes. *Science* 215, 1577-1585.
- Hollings M & Stone OM. 1973.** Some properties of chrysanthemum stunt, a virus with the characteristics of an uncoated ribonucleic acid. *Annals of Applied Biology* 74, 333-348.
- Hoofman R, Arts MJ, Shamloul AM, Van Zaayen A & Hadidi A. 1996.** Detection of chrysanthemum stunt viroid by reverse transcription-polymerase chain reaction and by tissue blot hybridization. *Acta Horticulturae* 432, 88-94.
- Horst RK, Langhans RW & Smith SH. 1977.** Effects of chrysanthemum stunt, chlorotic mottle, aspermy and mosaic on flowering and rooting of chrysanthemums. *Phytopathology* 67, 9-14.
- Hunter DE, Darling HM & Beale WL. 1969.** Seed transmission of potato spindle tuber virus. *American Potato Journal* 46, 247-250.
- Huson DH. 1998.** SplitsTree: analyzing and visualizing evolutionary data. *Bioinformatics* 14, 68-73.
- Hutchins CJ, Rathjen JP, Forster AC & Symons RH. 1986.** Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. *Nucleic Acids Research* 14, 3627-3640.

- Huttinga H, Mosch WHM & Treur A. 1987.** Comparison of bi-directional electrophoresis and molecular hybridisation methods to detect potato spindle tuber viroid and chrysanthemum stunt viroid. *EPPO Bulletin* 17, 37-43.
- Itaya A, Folimonow A, Matsuda Y, Nelson RS & Ding B. 2001.** *Potato spindle tuber viroid* as inducer of RNA silencing in infected tomato. *Molecular Plant-Microbe Interactions* 14, 1332-1334.
- Itaya A, Zhong X-H, Bundschuh R, Qi Y, Wang Y, Takeda R, Harris AR, Molina C, Nelson RS & Ding B. 2007.** A structured viroid RNA serves as a substrate for dicer-like cleavage to produce biologically active small RNAs but is resistant to RNA-induced silencing complex-mediated degradation. *Journal of Virology* 81, 2980-2994.
- Kastalyeva TB, Mozhaeva KA, Pisetskaya NF, Romanova SA & Trofimets LN. 1992.** The potato spindle tuber viroid and bringing potato into a healthy state. (in Russian) *Vestnik RASKHN* 3, 22-24.
- Kastalyeva TB, Mozhaeva KA, Thompson SM, Clark JR & Owens RA. 2007.** Recovery of four novel *Potato spindle tuber viroid* sequence variants from Russian seed potatoes. *Plant Disease* 91, 469.
- Keese P & Symons RH. 1985.** Domains in viroids: evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. *Proceedings of the National Academy of Science of the United States of America* 82, 4582-4586.
- Keese P, Visvader JE & Symons RH. 1988** Sequence variability in plant viroid RNAs. In: Domingo E, Holland JJ & Ahlquist P (eds). *RNA Genetics Vol. 3*. CRC Press, Boca Raton, Florida, USA, pp. 71-98.
- KNMI-daggegevens. nd.** Retrieved January 24, 2009, from <http://www.knmi.nl/klimatologie/daggegevens/download.html>.
- Koenraad H, Jodłowska A, Van Vliet A & Verhoeven K. 2009.** Detection of TCDVd and PSTVd in seeds of tomato. *Phytopathology* 99, S66.
- Kolonko N, Bannach O, Aschermann K, Hu K-H, Moors M, Schmitz M, Steger G & Riesner D. 2006.** Transcription of potato spindle tuber viroid by RNA polymerase II starts in the left terminal loop. *Virology* 347, 392-404.
- Koltunow AM & Rezaian MA. 1988.** Grapevine yellow speckle viroid: structural features of a new viroid group. *Nucleic Acids Research* 16, 849-864.
- Kowalska-Noordam A, Chrzanowska M & Skrzeczkowska S. 1987.** *Ziemniak 1886/87*, 71-92.
- Kryczynski S, Paduch-Cichal E & Skrzeczkowski LR. 1988.** Transmission of three viroids through seed and pollen of tomato plants. *Journal of Phytopathology* 121, 51-57.
- Kryczynski S, Stawiszynska A & Abuhliga TA. 1995.** The reaction of field-grown tomato cultivars to infection with potato spindle tuber viroid. *Phytopathologia Polonica* 22, 85-91.
- Lafontaine D, Beaudry D, Marquis P & Perreault JP. 1995.** Intra- and intermolecular nonenzymatic ligations occur within transcripts derived from the peach latent mosaic viroid. *Virology* 212, 705-709.
- Lebas BSM, Clover GRG, Ochoa-Corona FM, Elliott DR, Tang Z & Alexander BJR. 2005.** Distribution of *Potato spindle tuber viroid* in New Zealand glasshouse crops of capsicum and tomato. *Australasian Plant Pathology* 34, 129-133.
- Lee CR & Singh RP. 1972.** Enhancement of diagnostic symptoms of potato spindle tuber virus by manganese. *Phytopathology* 62, 516-520.
- Leontyeva JA. 1980.** Tomatoes and potato spindle tuber viroid. *Zashchita-Rastenii* 8, 22.
- Lesemann D-E, Preissel H-G & Verhoeven JThJ. 1996.** Detection of Colombian datura potyvirus and two unidentified potyviruses in *Brugmansia* hybrids. *Acta Horticulturae* 432, 346-353.

- Lima MI, Fonseca MEN, Flores R & Kitajima EW. 1994.** Detection of avocado sunblotch viroid in chloroplasts of avocado leaves by in situ hybridization. *Archives of Virology*, 138, 385-390.
- Ling KS & Bledsoe ME. 2009.** First report of *Mexican papita viroid* infecting greenhouse tomato in Canada. *Plant Disease* 93, 839.
- Ling KS, Verhoeven JThJ, Singh RP & Brown JK. 2009.** First report of *Tomato chlorotic dwarf viroid* in greenhouse tomatoes in Arizona, USA. *Plant Disease* 93, 1075.
- Liu FC, Chen RF & Chen YX. 1957.** Apple scar skin disease. Academia Sinica Printery, Beijing, China. (in Chinese) 43 pp.
- Loss P, Schmitz M, Steger G & Riesner D. 1991.** Formation of a thermodynamically metastable structure containing hairpin II is critical for infectivity of potato spindle tuber viroid RNA. *The EMBO Journal* 10, 719-727.
- Luckwill LC. 1963.** Apple chat fruit. In: Posnette AF (ed). *Virus diseases of apples and pears*. Technical Communication No. 30. Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England. pp 25-28.
- Mackie AE, McKirdy SJ, Rodoni B & Kumar S. 2002.** *Potato spindle tuber viroid* eradicated in Western Australia. *Australasian Plant Pathology* 31, 311-312.
- Malfitano M, Di Serio F, Covelli L, Ragazzino A, Hernández C & Flores R. 2003.** Peach latent mosaic viroid variants inducing peach calico (extreme chlorosis) contain a characteristic insertion that is responsible for this symptomatology. *Virology* 313, 492-501.
- Maniataki E, Tabler M & Tagris M. 2003.** Viroid RNA systemic spread may depend on the interaction of a 71-nucleotide bulged hairpin with the host protein VirP1. *RNA* 9, 346-354.
- Manzer FE & Merriam D. 1961.** Field transmission of the potato spindle tuber virus and virus X by cultivating and hilling equipment. *American Potato Journal* 38, 346-352.
- Markarian N, Li HW, Ding SW & Semancik JS. 2004.** RNA silencing as related to viroid induced symptom expression. *Archives of Virology* 149, 397-406.
- Martin WH. 1922.** "Spindle tuber", a new potato trouble. *Hints to potato growers*, New Jersey State Potato Association 3, no 8.
- Martínez-Soriano JP, Galindo-Alonso J, Maroon CJM, Yucel I, Smith DR & Diener TO. 1996.** Mexican papita viroid: putative ancestor of crop viroids. *Proceedings of the National Academy of Science of the United States of America* 93, 9397-9401.
- Mathews DH, Sabina J, Zuker M & Turner DH. 1999.** Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *Journal of Molecular Biology* 288, 911-940.
- Matsuura S, Matsushita Y, Kozuka R, Shimizu S & Tsuda S. 2010.** Transmission of *Tomato chlorotic dwarf viroid* (TCDVd) in tomato plants. *European Journal of Plant Pathology* 126, 111-115.
- Menzel W & Maiss E. 2000.** Detection of chrysanthemum stunt viroid (CSVd) in cultivars of *Argyranthemum frutescens* by RT-PCR-ELISA. *Journal of Plant Diseases and Protection* 107, 548-552.
- Mertelik J, Kloudova K, Cervena G, Necekalova J, Mikulkova H, Levkanicova Z, Dedic P & Ptacek J. 2009.** First report of *Potato spindle tuber viroid* (PSTVd) in *Brugmansia* spp., *Solanum jasminoides*, *Solanum muricatum* and *Petunia* spp. In the Czech Republic. *New Disease Reports* 19 (February-August).
- Mishra MD, Hammond RW, Owens RA, Smith DR & Diener TO. 1991.** Indian bunchy top disease of tomato plants is caused by a distinct strain of citrus exocortis viroid. *Journal of General Virology* 72, 1781-1785.
- Montagnier L. 1968.** The replication of viral RNA. *Symposium Society for General Microbiology* 18, 125-148.

- Motard J, Bolduc F, Thompson D & Perreault J. 2008.** The peach latent mosaic viroid replication initiation site is located at a universal position that appears to be defined by a conserved sequence. *Virology* 373, 362–375.
- Mühlbach HP & Sängler HL. 1979.** Viroid replication is inhibited by alpha-amanitin. *Nature* 278, 185–188.
- Mumford RA, Jarvis B & Skelton A. 2004.** The first report of *Potato spindle tuber viroid* (PSTVd) in commercial tomatoes in the UK. *Plant Pathology* 53, 242.
- Nakashima A, Hosokawa M, Maeda S & Yazawa S. 2007.** Natural infection of chrysanthemum stunt viroid in dahlia plants. *Journal of General Plant Pathology* 73, 225–227.
- Nakaune R & Nakano M. 2008.** Identification of a new apscaviroid from Japanese persimmon. *Archives of Virology* 153, 969–972.
- Navarro B, Silletti MR, Trisciuzzi VN & Di Serio F. 2009.** Identification and characterization of *Potato spindle tuber viroid* infecting tomato in Italy. *Journal of Plant Pathology* 91, 723–726.
- Navarro JA, Daròs JA & Flores R. 1999.** Complexes containing both polarity strands of avocado sunblotch viroid: Identification in chloroplasts and characterization. *Virology* 253, 77–85.
- Navarro JA & Flores R. 2000.** Characterization of the initiation sites of both polarity strands of a viroid RNA reveals a motif conserved in sequence and structure. *The EMBO Journal* 19, 2662–2670.
- Navarro JA, Vera A & Flores R. 2000.** A chloroplastic RNA polymerase resistant to tagetitoxin is involved in replication of *Avocado sunblotch viroid*. *Virology* 268, 218–225.
- Nei M & Kumar S. 2000.** *Molecular Evolution and Phylogenetics*. Oxford University Press, New York, USA.
- Nie X, Singh RP & Bostan H. 2005.** Molecular cloning, secondary structure, and phylogeny of three pospiviroids from ornamental plants. *Canadian Journal of Plant Pathology* 27, 592–602.
- Nixon T, Glover R, Mathews-Berry S, Daly M, Hobden E, Lanbourne C, Harju V & Skelton A. 2009.** *Columnea latent viroid* (CLVd) in tomato: the first report in the United Kingdom. *New Disease Reports* 19 (February–August).
- NPPO of Austria. 2008.** Situation of *Potato spindle tuber viroid* in Austria in 2008. EPPO Reporting Service, Vol. 9, No. 177. (<http://archives.eppo.org/EPPOReporting/2008/Rse-0809.pdf>)
- NPPO of France. 2002.** Phytosanitary incident: potato spindle tuber pospiviroid found and eradicated in France. EPPO Reporting Service, Vol. 9, No. 145. (<http://archives.eppo.org/EPPOReporting/2002/Rse-0209.pdf>)
- NPPO of Germany. 2000.** Situation of several quarantine pests in Germany in 1998. EPPO Reporting Service, Vol. 9, No. 138. (<http://archives.eppo.org/EPPOReporting/2000/Rse-0009.pdf>)
- Önelge N. 1997.** Direct nucleotide sequencing of citrus exocortis viroid (CEV). *Turkish Journal of Agriculture and Forestry* 21, 419–422.
- Owens RA. 2007.** Potato spindle tuber viroid: the simplicity paradox resolved? *Molecular Plant Pathology* 8, 549–560.
- Owens RA. 2008.** Viroids. In: Roossinck, MJ (ed). *Plant virus evolution*. Springer Verlag, Berlin, Germany, pp 83–108.
- Owens RA & Verhoeven JThJ. 2009.** Potato Spindle Tuber. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2009-0804-01.
- Owens RA Thompson SM & Steger G. 1991.** Effects of random mutagenesis upon potato spindle tuber viroid replication and symptom expression. *Virology* 185, 18–31.

- Owens RA, Steger G, Hu Y, Fels A, Hammond RW & Riesner D. 1996. RNA structural features responsible for potato spindle tuber viroid pathogenicity. *Virology* 222, 144-158.
- Owens RA, Blackburn M & Ding B. 2001. Possible involvement of the phloem lectin in long-distance viroid movement. *Molecular Plant Microbe Interaction* 14, 905-909.
- Owens RA, Girsova NV, Kromina KA, Lee IM, Mozhaeva KA & Kastalyeva T. 2009. Russian isolates of *Potato spindle tuber viroid* exhibit low sequence diversity. *Plant Disease* 93, 752-759.
- Palukaitis P. 1987. Potato spindle tuber viroid: investigation of the long-distance, intra-plant transport route. *Virology* 158, 239-241.
- Papaefthimiou I, Hamilton AJ, Denti MA, Baulcombe DC, Tsagris M & Tabler M. 2001. Replicating potato spindle tuber viroid RNA is accompanied by short RNA fragments that are characteristic of post-transcriptional gene silencing. *Nucleic Acids Research* 29, 2395-2400.
- Pelchat M, Grenier C & Perrault JP. 2002. Characterization of a viroid-derived RNA promoter for the DNA-dependent RNA polymerase from *Escherichia coli*. *Biochemistry* 41, 6561-6571.
- Pfannenstiel MA & Slack SA. 1980. Response of potato cultivars to infection by the potato spindle tuber viroid. *Phytopathology* 70, 922-926.
- Puchta H, Herold T, Verhoeven K, Roenhorst A, Ramm K, Schmidt-Puchta W & Sanger HL. 1990. A new strain of potato spindle tuber viroid (PSTVd-N) exhibits major sequence differences as compared to all other PSTVd strains sequenced so far. *Plant Molecular Biology* 15, 509-511.
- Qi Y & Ding B. 2003a. Inhibition of cell growth and shoot development by a specific nucleotide sequence in a noncoding viroid RNA. *The Plant Cell* 15, 1360-1374.
- Qi Y & Ding B. 2003b. Differential subnuclear localization of RNA strands of opposite polarity derived from an autonomously replicating viroid. *The Plant Cell* 15, 2566-2577.
- Querci M, Owens RA, Vargas C & Salazar LF. 1995. Detection of potato spindle tuber viroid in avocado growing in Peru. *Plant Disease* 79, 196-202.
- Querci M, Owens RA, Bartoli I, Lazarte V & Salazar LF. 1997. Evidence for heterologous encapsidation of potato spindle tuber viroid in particles of potato leafroll virus. *Journal of General Virology* 78, 1207-1211.
- Rackwitz HR, Rohde W & Sanger HL. 1981. DNA-dependent RNA polymerase II of plant origin transcribes viroid RNA into full-length copies. *Nature* 291, 297-301.
- Randles JW & Rodriguez MJB. 2003. Palm tree viroids: Coconut cadang cadang viroid. In: Hadidi A, Flores R, Randles JW & Semancik JS (eds). *Viroids*. CSIRO Publishing, Collingwood, Australia. pp 233-241.
- Raymer WB & O'Brien MJ. 1962. Transmission of potato spindle tuber virus to tomato. *American Potato Journal* 39, 401-408.
- Raymer WB & Diener TO. 1969. Potato spindle tuber virus: A plant virus with properties of a free nucleic acid. I Assay, extraction and concentration. *Virology* 37, 343-350.
- Riesner D. 1991. Viroids: from thermodynamics to cellular structure and function. *Molecular Plant-Microbe Interactions* 4, 122-131.
- Riesner D, Henco K, Rokohl U, Klotz G, Kleinschmidt AK, Domdey H, Jank P, Gross HJ & Sanger HL. 1979. Structure and structure formation of viroids. *Journal of Molecular Biology* 133, 85-115.
- Rodriguez MJB & Randles JW. 1993. Coconut cadang-cadang viroid (CCCVd) mutants associated with severe disease vary in both the pathogenicity domain and the central conserved region. *Nucleic Acids Research* 21, 2771.
- Rodio M-E, Delgado S, De Stradis A, G3mez MD, Flores R & Di Serio F. 2007. A viroid RNA with a specific structural motif inhibits chloroplast development. *The Plant Cell* 19, 3610-3626.

- Roenhorst JW & Verhoeven JThJ. 1995.** Virology. Verslagen en Mededelingen Plantenziektenkundige Dienst Wageningen 177 (Annual Report Diagnostic Centre 1994), pp 112-126.
- Roenhorst JW & Verhoeven JThJ. 1998.** Virology. Verslagen en Mededelingen Plantenziektenkundige Dienst Wageningen 193 (Annual Report Diagnostic Centre 1997), pp 106-123.
- Roenhorst JW & Verhoeven JThJ. 2000.** Virology. Verslagen en Mededelingen Plantenziektenkundige Dienst Wageningen 210 (Annual Report Diagnostic Centre 1999), pp 93-107.
- Roenhorst JW, Butôt RPT, Van der Heijden KA, Hooftman M & Van Zaayen A. 2000.** Detection of chrysanthemum stunt viroid and potato spindle tuber viroid by return-polyacrylamide gel electrophoresis. EPPO Bulletin 30, 453-456.
- Roenhorst JW, Jansen CCC, Kox LFF, De Haan EG, Van den Bovenkamp GW, Boonham N, Fisher T & Mumford RA. 2005.** Application of real-time RT-PCR for large-scale testing of potato for *Potato spindle tuber pospiviroid*. EPPO Bulletin 35, 133-140.
- Rzhetsky A & Nei M. 1992.** A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution 9, 945-967.
- Saitou N & Nei M. 1987.** The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406-425.
- Sänger H L & Ramm K. 1975.** Radioactive labelling of viroid-RNA. In: Markham R, Davies DR, Hopwood DA & Horne RW (eds). Modification of the information content of plant cells. Elsevier Publications, Amsterdam, The Netherlands. pp 229-252.
- Sänger HL, Ramm K, Domdey H, Gross HJ, Henco K & Riesner D. 1977.** Conversion of circular viroid molecules to linear strands. FEBS Letters 99, 117-122.
- Sanjuán R, Forment J & Elena SF. 2006.** In silico predicted robustness of viroid RNA secondary structure. I. The effect of single mutations. Molecular Biology and Evolution 23, 1427-1436.
- Sano T. 2003.** Hop viroids: *Hop stunt viroid*. In: Hadidi A, Flores R, Randles JW & Semancik JS (eds). Viroids. CSIRO Publishing, Collingwood, Australia. pp 207-212.
- Sano T, Candresse T, Hammond RW, Diener TO & Owens RA. 1992.** Identification of multiple structural domains regulating viroid pathogenicity. Proceedings of the National Academy of Sciences of the United States of America 89, 10104-10108.
- Sano T, Mimura R & Ohshima K. 2001.** Phylogenetic analysis of hop and grapevine isolates of hop stunt viroid supports a grapevine origin for hop stunt disease. Virus Genes 22, 53-59.
- Schindler IM & Mühlbach HP. 1992.** Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: a reevaluation. Plant Science 84, 221-229.
- Schnolzer M, Haas B, Ramm K, Hofmann H & Sänger HL. 1985.** Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV). The EMBO Journal 4, 2181-2190.
- Schrader O, Baumstark T & Riesner D. 2003.** A mini-RNA containing tetraloop, wobble-pair and loop E motifs of the central conserved region of potato spindle tuber viroid is processed into a minicircle. Nucleic Acids Research 31, 988-998.
- Schumacher J, Meyer N, Riesner D & Weidemann HL. 1986.** Diagnostic procedure for detection of viroids and viruses with circular RNAs by return-gel electrophoresis. Journal of Phytopathology 115, 332-343.
- Schultz ES & Folsom D. 1923.** Transmission, variation, and control of certain degeneration diseases of Irish potatoes. Journal of Agricultural Research 25, 43-118.

- Schwind N, Zwiebel M, Itaya A, Ding B, Wang MB, Krczal G & Wassenegger M. 2009.** RNA-mediated resistance to *Potato spindle tuber viroid* in transgenic tomato expressing a viroid hairpin construct. *Molecular Plant Pathology* 10, 459-469.
- Sdoodee R & Teakle DS. 1993.** Studies on the mechanism of transmission of pollen-associated tobacco streak ilarvirus virus by *Thrips tabaci*: a new method of plant virus transmission. *Plant Pathology* 42, 88-92.
- Seigner L, Kappen M, Huber C, Kistler M & Köhler D. 2008.** First trials for transmission of Potato spindle tuber viroid from ornamental Solanaceae to tomato using RT-PCR and an mRNA based internal positive control for detection. *Journal of Plant Diseases and Protection* 115, 97-101.
- Semancik JS, Magnuson DS & Weathers LG. 1973.** Potato spindle tuber disease produced by pathogenic RNA from citrus exocortis disease: evidence for the identity of the causal agents. *Virology* 52, 292-294.
- Serra P, Barbosa CJ, Daròs JA, Flores R & Duran-Vila N. 2008.** Citrus viroid V: Molecular characterization and synergistic interactions with other members of the genus Apscaviroid. *Virology* 370, 102-112.
- Shamloul AM, Hadidi A, Zhu SF, Singh RP & Sagredo B. 1997.** Sensitive detection of potato spindle tuber viroid using RT-PCR and identification of a viroid variant naturally infecting pepino plants. *Canadian Journal of Plant Pathology* 19, 89-96.
- Singh RP. 1970.** Seed transmission of potato spindle tuber virus in tomato and potato. *American Potato Journal* 47, 225-227.
- Singh RP. 1973.** Experimental host range of the potato spindle tuber 'virus'. *American Potato Journal* 50, 111-123.
- Singh RP & Dilworth AD. 2009.** *Tomato chlorotic dwarf viroid* in the ornamental plant *Vinca minor* and its transmission through tomato seed. *European Journal of Plant Pathology* 123, 111-116.
- Singh RP, Finnie RE & Bagnall RH. 1971.** Losses due to Potato spindle tuber viroid. *American Potato Journal* 48, 262-267.
- Singh RP, Boucher A & Seabrook JEA. 1988.** Detection of the mild strains of potato spindle tuber viroid from single true potato seed by return electrophoresis. *Phytopathology* 78, 663-667.
- Singh RP, Boucher A & Tai GCC. 1990.** High levels of viroid in tomato and potato plants inoculated with minimum amounts of of potato spindle tuber viroid. *Canadian Journal of Plant Pathology* 12, 11-15.
- Singh RP, Boucher A & Wang RG. 1991.** Detection, distribution and long-term persistence of potato spindle tuber viroid in true potato seed from Heilongjiang, China. *American Potato Journal* 68, 65-74.
- Singh RP, Lakshman DK, Boucher A & Tavantzis SM. 1992a.** A viroid from *Nematanthus wettsteinii* plants closely related to the Columnea latent viroid. *Journal of General Virology* 73, 2769-2774.
- Singh RP, Boucher A & Sommerville TH. 1992b.** Detection of potato spindle tuber viroid in the pollen and various parts of potato plant pollinated with viroid-infected pollen. *Plant Disease* 76, 951-953.
- Singh RP, Singh M, Boucher A & Owens RA. 1993.** A mild strain of potato spindle tuber viroid from China is similar to North American isolates. *Canadian Journal of Plant Pathology* 15, 134-138.
- Singh RP, Nie X & Singh M. 1999.** Tomato chlorotic dwarf viroid: an evolutionary link in the origin of pospiviroids. *Journal of General Virology* 80, 2823-2828.

- Singh RP, Ready KFM & Hadidi A. 2003.** Viroids in North America and global distribution of viroid diseases. In: Hadidi A, Flores R, Randles JW & Semancik JS (eds). *Viroids*. CSIRO Publishing, Collingwood, Australia. pp 255-264.
- Singh RP, Dilworth AD, Baranwal VK & Gupta KN. 2006.** Detection of *Citrus exocortis* viroid, *Iresine viroid* and *Tomato chlorotic dwarf viroid* in new ornamental host plants in India. *Plant Disease* 90, 1457.
- Skoric D, Conerly M, Szychowski JA & Semancik JS. 2001.** CEVd-induced symptom modification as a response to a host-specific temperature-sensitive reaction. *Virology* 280, 115-123.
- Sogo JM, Koller T & Diener TO. 1973.** Potato spindle tuber viroid. X. Visualization and size determination by electron microscopy. *Virology* 55, 70-80.
- Spieker RL. 1996a.** A viroid from *Brunfelsia undulata* closely related to the *Columnea* latent viroid. *Archives of Virology* 141, 1823-1832.
- Spieker RL. 1996b.** The molecular structure of *Iresine viroid*, a new viroid species from *Iresine herbstii* ('beefsteak plant'). *Journal of General Virology* 77, 2631-2635.
- Spieker RL, Marinkovic S & Sanger HL. 1996.** A viroid from *Solanum pseudocapsicum* related to the tomato apical stunt viroid. *Archives of Virology* 141, 1387-1495.
- Spiesmacher E, Muhlbach HP, Schnolzer M, Haas B & Sanger HL. 1983.** Oligomeric forms of potato spindle tuber viroid (PSTV) and of its complementary RNA are present in nuclei isolated from viroid-infected potato cells. *Bioscience Reports* 3, 767-774.
- Steyer S, Olivier T, Skelton A, Nixon T & Hobden E. 2009.** *Columnea latent viroid* (CLVd): first report in tomato in France. *New Disease Reports* 19 (February-August).
- Sun M, Siemsen S, Campbell W, Guzman P, Davidson R, Whitworth JL, Bourgoin T, Axford J, Schrage W, Leever G, Westra A, Marguadt S, El-Nashaar H, McMorran J, Gutbrod O, Wessels T & Coltman R. 2004.** Survey of potato spindle tuber viroid in seed potato growing areas of the United States. *American Journal of Potato Research* 81, 227-231.
- Swofford DL. 1993.** Phylogenetic analysis using parsimony (PAUP), Version 4.0.0. University of Illinois, Champaign, IL.
- Syller J, Marczewski W & Pawlowicz J. 1997.** Transmission by aphids of potato spindle tuber viroid encapsidated by potato leafroll luteovirus particles. *European Journal of Plant Pathology* 103, 285-289.
- Tabler M & Tsagris M. 2004.** Viroids: petite RNA pathogens with distinguished talents. *TRENDS in Plant Science* 9, 399-348.
- Takezaki N, Rzhetsky A & Nei M. 2004.** Phylogenetic test of the molecular clock and linearized trees. *Molecular Biology and Evolution* 12, 823-833.
- Tamura K, Nei M & Kumar S. 2004.** Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences of the United States of America* 101, 11030-11035.
- Tamura K, Dudley J, Nei M & Kumar S. 2007.** MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* 24, 1596-1599.
- Thompson JD, Higgins DG & Gibson TJ. 1994.** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673-4680.
- Tsagris EM, Martınez de Alba AE, Gozmanova M & Kalantidis K. 2008.** Viroids. *Cellular Microbiology* 11, 2168-2179.
- Van Dorst HJM & Peters D. 1974.** Some biological observations on pale fruit, a viroid-incited disease of cucumber. *Netherlands Journal of Plant Pathology* 80, 85-96.

- Van Wezenbeek P, Vos P, Van Boom J & Van Kammen A. 1982.** Molecular cloning and characterization of a complete DNA copy of potato spindle tuber viroid RNA. *Nucleic Acids Research* 10, 7947-7957.
- Verhoeven JThJ & Roenhorst JW. 2000.** Herbaceous test plants for the detection of quarantine viruses of potato. *EPPO Bulletin* 30, 463-467.
- Verhoeven JThJ & Roenhorst JW. 2010.** High stability of original predominant pospiviroid genotypes upon mechanical inoculation from ornamentals to potato and tomato. *Archives of Virology* (doi: 10.1007/s00705-009-0572-9).
- Verhoeven JThJ, Arts MSJ, Owens RA & Roenhorst JW. 1998.** Natural infection of petunia by chrysanthemum stunt viroid. *European Journal of Plant Pathology* 104, 383-386.
- Verhoeven JThJ, Jansen CCC, Willemsen TM, Kox LFF, Owens RA & Roenhorst JW. 2004.** Natural infections of tomato by *Citrus exocortis viroid*, *Columnea latent viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. *European Journal of Plant Pathology* 110, 823-831.
- Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2006a.** First report of *Tomato apical stunt viroid* in tomato in Tunisia. *Plant Disease* 90, 528.
- Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2006b.** First report of *Potato virus M* and *Chrysanthemum stunt viroid* in *Solanum jasminoides*. *Plant Disease* 90, 1359.
- Verhoeven JThJ, Jansen CCC, Roenhorst JW, Steyer S & Michelante D. 2007a.** First report of *Potato spindle tuber viroid* in tomato in Belgium. *Plant Disease* 91, 1055.
- Verhoeven JThJ, Jansen CCC, Werkman AW & Roenhorst JW. 2007b.** First report of *Tomato chlorotic dwarf viroid* in *Petunia hybrida* from the United States of America. *Plant Disease* 91, 324.
- Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2008a.** First report of pospiviroids infecting ornamentals in the Netherlands: *Citrus exocortis viroid* in *Verbena* sp., *Potato spindle tuber viroid* in *Brugmansia suaveolens* and *Solanum jasminoides*, and *Tomato apical stunt viroid* in *Cestrum* sp. *Plant Pathology* 57, 399.
- Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2008b.** *Streptosolen jamesonii* 'Yellow', a new host plant of *Potato spindle tuber viroid*. *Plant Pathology* 57, 399.
- Verhoeven JThJ, Jansen CCC, Roenhorst JW, Steyer S, Schwind N & Wassenegger M. 2008c.** First report of *Solanum jasminoides* infected by *Citrus exocortis viroid* in Germany and the Netherlands and *Tomato apical stunt viroid* in Belgium and Germany. *Plant Disease* 92, 973.
- Verhoeven JThJ, Botermans M, Roenhorst JW, Westerhof J & Meekes ETM. 2009a.** First report of *Potato spindle tuber viroid* in Cape gooseberry (*Physalis peruviana*) from Turkey and Germany. *Plant Disease* 93, 316.
- Verhoeven JThJ, Jansen CCC, Roenhorst JW, Flores R & De la Peña M. 2009b.** *Pepper chat fruit viroid*: biological and molecular properties of a proposed new species of the genus Pospiviroid. *Virus Research* 144, 209-214.
- Verhoeven JThJ, Jansen CCC, Botermans M & Roenhorst JW. 2010.** Epidemiological evidence that vegetatively propagated, solanaceous plant species act as sources of *Potato spindle tuber viroid* inoculum for tomato. *Plant Pathology* 59, 3-12.
- Visvader JE & Symons RH. 1985.** Eleven new sequence variants of citrus exocortis viroid and the correlation of sequence with pathogenicity. *Nucleic Acids Research* 13, 2907-2920.
- Visvader JE & Symons RH. 1986.** Replication of in vitro constructed viroid mutants: Location of the pathogenicity-modulating domain of citrus exocortis viroid. *The EMBO Journal* 5, 2051-2055.
- Walter B. 1987.** Tomato apical stunt. In: Diener TO (ed). *The viroids*. Plenum Press, New York, USA. pp 321-326.

- Walter B, Thouvenal JC & Fauquet C. 1980.** Les virus de la tomate en Cote d'Ivoire. *Annales de Phytopathologie* 12, 259-275.
- Wang M-B, Bian X-Y, Wu L-M, Liu L-X, Smith NA, Isenegger D, Wu R-M, Masuta C, Vance VB, Watson JM, Rezaian A, Dennis ES & Waterhouse PM. 2004.** On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. *Proceedings of the National Academy of Sciences of the United States of America* 101, 3275-3280.
- Wassenegger M, Spieker RL, Thalmeir S, Gast FU, Riedel L & Sanger HL. 1996.** A single nucleotide substitution converts potato spindle tuber viroid (PSTVd) from a non-infectious to an infectious RNA for *Nicotiana tabacum*. *Virology* 226, 191-197.
- Weathers LG. 1964.** Nitrogen as a factor in the development of exocortis of citrus. *Phytopathology* 54, 968-969.
- Weller SA, Elphinstone JG, Smith NC, Boonham N & Stead DE. 2000.** Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied and Environmental Microbiology* 66, 2853-2858.
- Zhong X, Leontis N, Qian S, Itaya A, Qi Y, Boris-Lawrie K & Ding B. 2006.** Tertiary structural and functional analyses of a viroid RNA motif by isostericity matrix and mutagenesis reveal its essential role in replication. *Journal of Virology* 80, 8566-8581.
- Zhong X, Archual AJ, Amin AA & Ding B. 2008.** A genomic map of viroid RNA motifs critical for replication and systemic trafficking. *The Plant Cell* 20, 35-47.
- Zhu Y, Green L, Woo YM, Owens R & Ding B. 2001.** Cellular basis of potato spindle tuber viroid systemic movement. *Virology* 279, 69-77.
- Zuker M. 2003.** Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* 31, 3406-3415.

Summary

Viroids are the smallest infectious agents known. They only consist of a single-stranded circular RNA, too small to code for any proteins. Despite their small size, viroids cause diseases in various fruit, ornamental and vegetable crops. *Potato spindle tuber viroid* (PSTVd), the type species of the genus *Pospiviroid*, may cause serious diseases in potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*) and therefore, it is regulated as a quarantine 'organism' in most countries. However, also other, non-regulated pospiviroids have been isolated from severely affected tomato crops in recent years.

Since 1988 viroid infections have occasionally been detected by return-polyacrylamide gel electrophoresis in tomato crops in the Netherlands. The identity of the viroids, however, could not be determined with this method. Initially, only the first isolate from 1988 was sequenced and identified as PSTVd. All viroid infections detected since were not identified until 'Reverse Transcription-Polymerase Chain Reaction' (RT-PCR) and sequencing became commonly available at the beginning of this century. This thesis describes the identification of viroids detected in tomato from 1988 to 2009 and the characterization of a novel viroid from pepper (*Capsicum annuum*) using newly developed sensitive and specific RT-PCR approaches, as well as several epidemiological aspects such as distribution, forensics and possible transmission routes.

Two primer sets for RT-PCR were developed for the detection and (partial) identification of all pospiviroids. Using these primer sets and subsequent sequencing of the PCR products enabled identification of all pospiviroids found in tomato in the Netherlands, i.e. *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd) and PSTVd, respectively. In addition, *Tomato apical stunt viroid* (TASVd) and *Tomato chlorotic dwarf viroid* (TCDVd) were identified in tomato samples from abroad. Also a new viroid was identified in pepper plants that showed growth reduction and substantially smaller fruits. Both molecular and biological studies were performed to characterize this new viroid, for which the name *Pepper chat fruit viroid* (PCFVd) is proposed.

To learn more about the epidemiology of the pospiviroids from tomato attempts were made to trace the origins of the infections. The two outbreaks of PSTVd in 1988 could be associated with PSTVd-infected plants of pepino (*Solanum muricatum*) from New Zealand. For the remaining infections in the Netherlands, however, no sources of inoculum could be identified since they were not related to either contaminated seeds or plants for planting. Because the most frequently identified viroid, CLVd, had only been reported from symptomlessly infected ornamental plant species, surveys were started to screen for the presence of

pospiviroids in these crops. Testing with the primer sets that enable detection and identification revealed the presence of different pospiviroids in various symptomless ornamental plants species.

Since PSTVd, having a quarantine status in the EU, was found in plants of *Brugmansia suaveolens* and *Solanum jasminoides*, random samples from all commercially produced lots of both species in the Netherlands had to be screened for this viroid. A large number of lots appeared infected, indicating a huge potential of PSTVd inoculum for potato and tomato crops. However, this does not necessarily imply that these ornamental plants indeed acted as sources of inoculum in the past. Therefore, phylogenetic analyses were performed to elucidate potential relations between the PSTVd infections in tomato and other plant species. These analyses showed that PSTVd genotypes mainly group according to the plant species they were isolated from. There were clusters for PSTVd sequences from all vegetatively propagated species *Brugmansia* spp., *S. jasminoides* and potato, and there was a cluster of sequences from Cape gooseberry (*Physalis peruviana*) a plant species propagated by both cuttings and seeds. The formation of these clusters can be understood since the vegetative propagation of infected plants guarantees transmission of the viroid to all resulting progeny and as a consequence causes permanent sources of infection. The PSTVd genotypes from tomato, however, did not form a separate cluster but were distributed among the other clusters. This can be explained by the fact that infected tomato plants do not act as permanent sources of infection because they are generatively propagated and implies that infections must be introduced in each new crop. Therefore, it was concluded that vegetatively propagated crops (mainly ornamentals) act as sources of inoculum for tomato, and not vice versa.

This conclusion was further supported by the successful mechanical inoculation of pospiviroids from ornamentals to potato and tomato. Moreover, sequencing of the predominant pospiviroid genotypes first in the ornamentals and, upon transmission, in potato and tomato showed a high stability of these genotypes. Also after repeated transmission in both vegetable crops, only a few changes of predominant genotypes were identified. These results further substantiated the conclusion of the phylogenetic analyses in that these viroids are genetically stable upon transmission into other hosts.

The successful transmission of PSTVd by fingers and razor blades showed their potential role in the spread of the pospiviroids from one crop to another. Transmission from ornamentals to tomato by fingertips even was successful after a latency period of two hours and by contaminated razor blades after a single slash. Transmission was much more efficient at 25 °C than at 15 °C. In addition, tomato was found to be more susceptible to PSTVd than *B. suaveolens*, *S. jasminoides* and potato. Finally, *S. jasminoides* was a better source of inoculum than *B. suaveolens*. These results demonstrate that mechanical transmission may play a role in

pospiviroid epidemiology. Furthermore, they explain that pospiviroid outbreaks occur more frequently in tomato than in potato.

In conclusion, in this thesis all pospiviroids found tomato were identified and a new viroid in pepper was characterized with the help of two newly designed primers sets. In addition, the role of ornamental plants as sources of inoculum was shown, and mechanical transmission was found to be a realistic pathway for transfer of pospiviroids from ornamentals to potato and tomato. Finally, this study provides new knowledge on pospiviroids and their epidemiology, which could contribute to evaluate the regulation of pospiviroids.

Samenvatting

Viroïden zijn de kleinste ziekteverwekkers van planten. Ze bestaan slechts uit een enkelstrengs circulair RNA-molecuul, dat te klein is om zelfs voor één eiwit te coderen. Desondanks veroorzaken viroïden ziekten in verschillende bloemisterij-, fruit- en groentegewassen. Het aardappelspindelknolviroïde (*Potato spindle tuber viroid*; PSTVd), de 'type species' van het geslacht *Pospiviroid*, kan ernstige ziekten veroorzaken in aardappel (*Solanum tuberosum*) en tomaat (*Solanum lycopersicum*). Daarom heeft dit viroïde in veel landen een quarantainestatus. De laatste jaren zijn er echter ook andere, niet-gereguleerde pospiviroïden aangetroffen in ernstig aangetaste tomatengewassen.

Sinds 1988 zijn er in Nederland incidenteel viroïde-infecties gedetecteerd in tomaat met de 'Return-Polyacrylamide Gel Electrophoresis'-techniek. Deze techniek kan wel pospiviroïden detecteren, maar niet identificeren. Aanvankelijk is alleen het eerst gevonden pospiviroïde-isolaat op basis van de nucleotide-sequentie geïdentificeerd als PSTVd. Later werd de identiteit van de overige viroïde-isolaten bepaald, nadat de 'Reverse Transcription-Polymerase Chain Reaction' (RT-PCR) algemeen beschikbaar was gekomen omstreeks 2000. Dit proefschrift beschrijft de identificatie van de viroïden bij tomaat, die zijn gedetecteerd tussen 1988 en 2009, en de karakterisatie van een nieuw viroïde bij paprika (*Capsicum annuum*). Daarnaast zijn epidemiologische aspecten onderzocht, waaronder de herkomst en verspreiding van deze pospiviroïden.

Voor de detectie van alle bekende pospiviroïden werden twee primer sets voor RT-PCR ontwikkeld. Hiermee konden, door het 'sequencen' van de PCR-producten, alle in Nederland in tomaat gevonden pospiviroïden worden geïdentificeerd als respectievelijk *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd) en PSTVd. Daarnaast werden het *Tomato apical stunt viroid* (TASVd) en *Tomato chlorotic dwarf viroid* (TCDVd) geïdentificeerd in tomatenmonsters uit het buitenland. Bovendien werd met deze primers de eerste stap gezet naar de identificatie van een nieuw viroïde dat bij paprika groeiremming van planten en vruchten veroorzaakt. Het viroïde is zowel moleculair als biologisch nader gekarakteriseerd. Als naam is voorgesteld: *Pepper chat fruit viroid* (PCFVd).

Om meer inzicht in de epidemiologie van de pospiviroïden van tomaat te krijgen is geprobeerd om de infectiebronnen te achterhalen. Beide uitbraken van PSTVd in 1988 konden in verband worden gebracht met door PSTVd-geïnfecteerde pepino-planten uit Nieuw Zeeland. Voor de overige infecties in Nederland konden geen infectiebronnen worden achterhaald, omdat geen verband kon worden gelegd met geïnfecteerde partijen tomatenzaad of -planten. Daar CLVd tot dan toe alleen was gerapporteerd in drie symptoomloos geïnfecteerde bloemisterijgewassen, zijn

vervolgens surveys uitgevoerd in de bloemisterij. Hierbij werden door toetsing met de ontwikkelde primer sets meerdere pospiviroïden vastgesteld in verschillende bloemisterijgewassen. Geen van de geïnfekteerde planten toonde viroïde-symptomen.

PSTVd werd aangetroffen in planten van *Brugmansia suaveolens* en *Solanum jasminoides*. Vanwege de quarantainestatus van dit viroïde in de EU, moesten alle commerciële teelten van deze gewassen in Nederland worden getoetst. Hierbij bleek een groot aantal partijen geïnfekteerd. Dit betekende dat er op dat moment een groot aantal PSTVd-infectiebronnen aanwezig was. Hieruit kon echter niet zonder meer worden afgeleid dat deze gewassen in het verleden ook werkelijk als infectiebron hadden gefungeerd voor tomaat. Door fylogenetische analyse konden echter verbanden worden gelegd tussen PSTVd-infecties in tomaat en enkele andere plantensoorten. Het bleek namelijk dat de genotypen van PSTVd clusters vormden, overeenkomstig de plantensoort waaruit ze waren geïsoleerd. Zo waren er clusters met PSTVd-sequenties van alle vegetatief vermeerderde waardplanten: *Brugmansia* spp., *S. jasminoides* en aardappel. Daarnaast was er een cluster met sequenties van *Physalis peruviana*, een plantensoort die zowel via stek als zaad wordt vermeerderd. De vorming van deze clusters is logisch omdat vegetatieve vermeerdering van geïnfekteerde planten altijd leidt tot overdracht van het viroïde naar de nakomelingen, die bijgevolg permanente infectiebronnen zijn. De PSTVd-genotypen uit tomaat vormden echter geen afzonderlijk cluster, maar waren verdeeld over de andere clusters. Een verklaring hiervoor is dat tomatenplanten via zaad worden vermeerderd hetgeen betekent dat infecties in elke teelt opnieuw moeten worden geïntroduceerd. Op basis van deze analyse is geconcludeerd dat vegetatief vermeerderde gewassen (vooral bloemisterijgewassen) fungeren als infectiebron voor tomaat en niet omgekeerd.

Deze conclusie werd verder onderbouwd door de succesvolle mechanische overdracht van pospiviroïden van bloemisterijgewassen naar aardappel en tomaat. Daarbij bleek het meest voorkomende pospiviroïde-genotype zeer stabiel, zowel in de bloemisterijgewassen als na overdracht in aardappel en tomaat. Zelfs na herhaalde overdracht werden in de twee laatstgenoemde gewassen slechts enkele veranderingen waargenomen in het meest voorkomende genotype. Deze resultaten ondersteunen de conclusie van het fylogenetische onderzoek.

De geslaagde overdracht van PSTVd via besmette vingers en scheermesjes toonde aan dat deze wijze van overdracht in de praktijk een rol kan spelen in de overdracht van pospiviroïden van het ene naar het andere gewas. De overdracht via vingers bleek mogelijk tot tenminste twee uur na contact met een geïnfekteerde plant. Tevens slaagde overdracht via scheermesjes naar tomaat in de meeste gevallen na één enkele snede. Zoals verwacht was de overdracht bij 25 °C efficiënter dan bij 15 °C. Tomaat bleek vatbaarder voor PSTVd dan aardappel, *B. suaveolens*, en *S. jasminoides*. Tot slot bleek *S. jasminoides* een betere infectiebron dan *B. suaveolens*.

Deze resultaten tonen aan dat mechanische overdracht een rol kan spelen in de epidemiologie van pospiviroïden en verklaren bovendien waarom uitbraken van pospiviroïden vaker voorkomen in tomaat dan in aardappel.

Dit proefschrift laat zien dat alle pospiviroïden bij tomaat kunnen worden geïdentificeerd via RT-PCR met twee nieuw ontwikkelde primer sets. Deze tests leverden tevens de aanzet tot de karakterisatie van een nieuw viroïde in paprika. Daarnaast werd aangetoond dat bloemsterijgewassen kunnen fungeren als infectiebronnen van pospiviroïden, waarbij mechanische overdracht naar aardappel en tomaat reëel is. Deze nieuwe kennis over pospiviroïden levert een bijdrage aan een eventuele (her)evaluatie betreffende de huidige wet- en regelgeving ten aanzien van deze ziekteverwekkers.

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Doing a PhD study is a lonely job, but you are not doing so alone! Many people have contributed greatly. The late professor Rob Goldbach, my promotor in the first years, stimulated me taking up a PhD study and inspired me with his enthusiasm. After his tragic accident professor Just Vlak took over smoothly. He timely provided all necessary support to finish the study on schedule.

My copromotor dr. Annelien Roenhorst I would like to thank for her great support during the preparation of my thesis but also for managing of our virological unit from 1989 when she started as my group leader. From the very beginning we cooperated intensively. Her scientific approach, perseverance and positive attitude developed my skills to start a PhD study some years ago. In addition, Annelien was a great help in preparing publications. Annelien, my sincere thanks.

My former Head of Department National Reference Centre (NRC) within the Plant Protection Service (PPS), dr.ir. Nicolette Klijn, reacted positively at my request to start a PhD study. Consequently, she provided the necessary facilities to perform all studies. The current Head ir. Mariëtte Edema continued this support, which enabled me to conclude my study timely. I am indebted to both.

Officially, my PhD study started 1st January 2007. However, I have been working on pospiviroids periodically since the beginning of my career. Initially, I was involved in testing of potato breeding material for *Potato spindle tuber viroid* (PSTVd) and other quarantine 'organisms'. From this period I would like to thank the late ir. Bram Treur, Theo van de Berg, Jacques Budding, Olga Cleij, Marjan van Houwelingen and Marjon van Vliet. Later I worked on the detection of pospiviroids in tomato and contributed to eradication of the respective outbreaks. For this period I would like to thank Marianne van der Blom, the late ing. Age Visser, ing. Jan Heres and ir. Marc Roosjen. From autumn 2006 we succeeded to eradicate PSTVd in many lots of ornamental plants within half a year, thanks to a close cooperation with many people from various Departments of the PPS. Despite the hectic of this period I keep good memories collaborating with many colleagues, especially Jan Heesters, Jan Hendriks, ir. Bram de Hoop, ir. Jeroen Kavelaars, dr. Irene Koomen, Maarten van Merriënboer, ir. Jan-Arie Nugteren, ing. Dolf Smid, ir. Laurens Smits and drs. mr. Pieter de Vries. I am still proud of our joint results.

During my study a lot of (real-time) RT-PCRs were run and a lot of sequences were determined in the laboratories of our Department. Special thanks are for ing. Claudia Jansen who coordinated a lot of this work and contributed a major part herself. Furthermore, I would like to thank all people who contributed to this work, i.e. dr.ir. Maria Bergsma-Vlami, Maureen Bruil, dr. Linda Kox, ir. Dave Lammers, ir. Sonia Somovilla-Carrasco, Jantine van Veldhuizen, ing. Joris Voogd and ir. Doriet

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As shown above many people have contributed a lot to the success of my PhD study but one of the main prerequisites to conclude a PhD study successfully is having good fellow colleagues. I have been very lucky in this matter. Ir. Marleen Botermans supported me with the phylogenetic analyses and contributed greatly to the diagnosis of samples that kept coming during my study. Lester Hüner very accurately did a lot of inoculations, prepared many samples for PCR testing, took care of our pospiviroid collection and was always ready to help on whatever was needed. Ir. Arjen Werkman put all my thesis documents in InDesign, which saved me a lot of time and greatly contributed to the layout of my thesis.

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A solid family basis is the last but also the main prerequisite for a successful PhD study. I am very grateful to my late parents for the opportunity they gave me to take up studies, which included that I would not succeed my father as a farmer. I profoundly thank my wife Mieke van Hooft for having a happy marriage, which offers ample opportunities for individual development but at the same time functions as a safe harbour. Finally, I thank my two beautiful sons, Lennart and Marco, who have grown up to independent individuals but still show their respect for the education given by Mieke and me. In such a benifical environment one can only flourish!

Curriculum vitae



J.Th.J. (Ko) Verhoeven, born June 22 in 1954, grew up at his parental farm in Bern, the Netherlands. In 1974 he received his Atheneum diploma from the Dr. Mollercollege in Waalwijk. After fulfilling his military service he continued his studies at the Hogere Agrarische School of 's-Hertogenbosch. In 1979 he received a BSc degree in Agricultural Plant Production. In the same year he was appointed at the Plant Protection Service in the Netherlands, where he is still employed. In the first two years the author attended a general training on plant pathology for all employees working in the Field Service. This included theoretical training at the Head Office in Wageningen

and internships of two to three months at the Regional Offices in Barendrecht, Groningen and Zaltbommel. Since 1981 he has worked at the Head Office in Wageningen. Till 1983 he coordinated the post-entry quarantine programme in potato and supervised breeding companies working with quarantine organisms. Thereafter, he was responsible for obtaining (selection, heat-treatment and testing) and maintaining virus-tested nuclear-stock material of woody crops. In addition, he diagnosed viral- and non-parasitic diseases of nursery stock, fruit tree crops and perennials. Since 1989 he specialised in diagnosing diseases caused by plant viruses, viroids and phytoplasmas. In addition, he contributed to research on epidemiology, detection and identification methods, and on taxonomy of viruses and viroids. He also trained foreign employees on virus diagnosis, contributed to Pest Risk Analyses and addressed virus-related disputes concerning im- and export of plants.

From 1989 till 1998 he was a member of the Board of the Netherlands Circle of Plant Virology and now is still secretary of its Commission on Nomenclature of Dutch virus names. He was also a member of the Board of the International Working Group on Legume and Vegetable Viruses from 2002 till 2008 and acted as its secretary during the last three years. From 2006 he is chairing the Nomenclature Commission of the Royal Netherlands Society of Plant Pathology. During the work on his thesis he collaborated with the Laboratory of Virology of the Wageningen University.

Account

Hassani-Mehraban A, Botermans M, Verhoeven JThJ, Meekes E, Saaier J, Goldbach R & Kormelink R. 2010. A distinct tospovirus causing necrotic streak on *Alstroemeria* sp. in Columbia. Archives of Virology 155, 423-428.

Verhoeven JThJ, Jansen CCC, Botermans M & Roenhorst JW. 2010. Epidemiological evidence that vegetatively propagated, solanaceous plant species act as sources of *Potato spindle tuber viroid* inoculum for tomato. Plant Pathology 59, 3-12.

Verhoeven JThJ & Roenhorst JW. 2010. High stability of original predominant pospiviroid genotypes upon mechanical inoculation from ornamentals to potato and tomato. Archives of Virology 155, 269-274.

Botermans M, Verhoeven JThJ, Jansen CCC, Roenhorst JW, Stijger CCMM & Pham KTK. 2009. First Report of *Tomato yellow leaf curl virus* in Tomato in the Netherlands. Plant Disease 93, 1073.

Koenraadt H, Jodlowska A, Van Vliet A & Verhoeven K. 2009. Detection of TCDVd and PSTVd in seeds of tomato. Phytopathology 99, S66.

Ling KS, Verhoeven JThJ, Singh RP & Brown JK. 2009. First report of *Tomato chlorotic dwarf viroid* in greenhouse tomatoes in Arizona, USA. Plant Disease 93, 1075.

Owens RA & Verhoeven JThJ. 2009. Potato Spindle Tuber. The Plant Health Instructor. DOI: 10.1094/PHI-I-2009-0804-01.

Verhoeven JThJ, Botermans M, Roenhorst JW, Westerhof J & Meekes ETM. 2009. First report of *Potato spindle tuber viroid* in Cape gooseberry (*Physalis peruviana*) from Turkey and Germany. Plant Disease 93, 316.

Verhoeven JThJ, Jansen CCC, Roenhorst JW, Flores R & De la Peña M. 2009. *Pepper chat fruit viroid*: biological and molecular properties of a proposed new species of the genus *Pospiviroid*. Virus Research 144, 209-214.

De Hoop MB, Verhoeven JThJ & Roenhorst JW. 2008. Phytosanitary measures in the European Union: a call for more dynamic risk management allowing more focus on real pest risks. Case study: *Potato spindle tuber viroid* (PSTVd) on ornamental Solanaceae in Europe. EPPO Bulletin 38, 510-515.

Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2008. First report of pospiviroids infecting ornamentals in the Netherlands: *Citrus exocortis* viroid in *Verbena* sp., *Potato spindle tuber viroid* in *Brugmansia suaveolens* and *Solanum jasminoides*, and *Tomato apical stunt viroid* in *Cestrum* sp. Plant Pathology 57, 399.

Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2008. *Streptosolen jamesonii* 'Yellow', a new host plant of *Potato spindle tuber viroid*. Plant Pathology 57, 399.

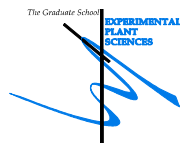
Verhoeven JThJ, Jansen CCC, Roenhorst JW, Steyer S, Schwind N & Wassenegger M. 2008. First report of *Solanum jasminoides* infected by *Citrus exocortis* viroid in Germany and the Netherlands and *Tomato apical stunt viroid* in Belgium and Germany. Plant Disease 92, 973.

Verhoeven JThJ, Roenhorst JW & Jongedijk GP. 2008. Occurrence and control of *Plum pox virus* in the Netherlands. Acta Horticulturae 781, 197-202.

Verhoeven JThJ, Jansen CCC, Roenhorst JW, Steyer S & Michelante D. 2007. First report of *Potato spindle tuber viroid* in tomato in Belgium. Plant Disease 91, 1055.

- Verhoeven JThJ, Jansen CCC, Werkman AW & Roenhorst, JW. 2007.** First report of *Tomato chlorotic dwarf viroid* in *Petunia hybrida* from the United States of America. *Plant Disease* 91, 324.
- Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2006.** First report of *Potato virus M* and *Chrysanthemum stunt viroid* in *Solanum jasminoides*. *Plant Disease* 90, 1359.
- Verhoeven JThJ, Jansen CCC & Roenhorst JW. 2006.** First report of *Tomato apical stunt viroid* in tomato in Tunisia. *Plant Disease* 90, 528.
- Verhoeven JThJ, Roenhorst JW, Lesemann D-E & Koenig R. 2006.** Identification of tombusviruses isolated from *Limonium sinuatum* on the basis of host range, serological and molecular studies. *Acta Horticulturae* 722, 201-207.
- Koenig R, Verhoeven JThJ, Fribourg CE, Pfeilstetter E & Lesemann D-E. 2004.** Evaluation of various species demarcation criteria in attempts to classify ten new tombusvirus isolates. *Archives of Virology* 149, 1733-1744.
- Verhoeven JThJ, Jansen CCC, Willemsen TM, Kox LFF, Owens RA & Roenhorst JW. 2004.** Natural infections of tomato by *Citrus exocortis viroid*, *Columnnea latent viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. *European Journal of Plant Pathology* 110, 823-831.
- Verhoeven JThJ & Roenhorst JW. 2003.** Detection of a broad range of potato viruses in a single assay by mechanical inoculation of herbaceous test plants. *EPPO Bulletin* 33, 305-311.
- Verhoeven JThJ, Roenhorst JW, Lesemann D-E, Segundo E, Velasco L, Ruiz L, Janssen D & Cuadrado IM. 2003.** *Southern bean mosaic virus* the causal agent of a new disease of *Phaseolus vulgaris* beans in Spain. *European Journal of Plant Pathology* 109, 935-941.
- Verhoeven JThJ, Van der Vlugt RAA & Roenhorst JW. 2003.** High similarity between tomato isolates of *Pepino mosaic virus* suggests a common origin. *European Journal of Plant Pathology* 109, 419-425.
- Verhoeven JThJ, Willemsen TM, Roenhorst JW & Van der Vlugt RAA. 2003.** First report of *Tomato infectious chlorosis virus* in tomato in Indonesia. *Plant Disease* 87, 872.
- Van der Vlugt RAA, Cuperus C, Vink J, Stijger CCMM, Lesemann D-E, Verhoeven JThJ & Roenhorst JW. 2002.** Identification and characterization of *Pepino mosaic virus* in tomato. *EPPO Bulletin* 32, 503-508.
- Verhoeven JThJ, Ding YM, Lesemann D-E, Van der Vlugt RAA & Roenhorst JW. 2002.** Virus infections in *Gentiana* species. *Acta Horticulturae* 568, 61-67.
- Verhoeven JThJ & Roenhorst JW. 2000.** Herbaceous test plants for the detection of quarantine viruses of potato. *EPPO Bulletin* 30, 463-467.
- Verhoeven JThJ, Arts MSJ, Owens RA & Roenhorst JW. 1998.** Natural infection of petunia by chrysanthemum stunt viroid. *European Journal of Plant Pathology* 104, 383-386.
- Verhoeven JThJ, De Haas AM & Roenhorst JW. 1998.** Outbreak and eradication of plum pox potyvirus in the Netherlands. *Acta Horticulturae* 472, 407-411.
- Verhoeven JThJ, Lesemann D-E & Roenhorst JW. 1996.** First report of Colombian datura potyvirus in tomato. *European Journal of Plant Pathology* 102, 895-898.
- Verhoeven JThJ, Bouwen I & Roenhorst JW. 1995.** A new flower breaking tobamovirus of *Streptocarpus*. *European Journal of Plant Pathology* 101, 311-318.
- Verhoeven JThJ & Roenhorst JW. 1994.** Tomato spotted wilt virus: ecological aspects in ornamental crops in the Netherlands from 1989 up to 1991. *Acta Horticulturae* 377, 175-182.

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Wageningen University

1) Start-up phase <ul style="list-style-type: none"> ▶ First presentation of your project ▶ Writing or rewriting a project proposal A proposal was written in december 2006 and has been adapted since when appropriate. Title: research topics PhD ▶ Writing a review or book chapter R.A. Owens & J.Th.J. Verhoeven (submitted) <i>Potato spindle tuber viroid</i>. Book chapter in Ninth Report of the ICTV ▶ MSc courses ▶ Laboratory use of isotopes 	<div style="text-align: right;"><i>date</i></div> <div style="text-align: right;">Dec 2006</div> <div style="text-align: right;">Nov 2009</div>
<i>Subtotal Start-up Phase</i>	
6.0 credits*	
2) Scientific Exposure <ul style="list-style-type: none"> ▶ EPS PhD Student Days ▶ EPS Theme Symposia ▶ NWO Lunteren days and other National Platforms NWO-ALW meeting 'Experimental Plant Sciences', Lunteren ▶ Seminars (series), workshops and symposia Annual meeting Dutch Circle of Plant Virology, Wageningen, The Netherlands 'Viroldendag', organised by Plantenziektenkundige Dienst and Naktuinbouw, Roelofarendsveen, The Netherlands Annual meeting Royal Netherlands Society of Plant Pathology, Wageningen, The Netherlands WEES Seminar dr. Santiago Elena Laboratory of Virology Seminar dr. Renato de Resende ▶ Seminar plus ▶ International symposia and congresses (from 2007) EU Workshop on apple proliferation phytoplasma, Prague, Czech republic 10th International Plant Virus Epidemiology Symposium, Hyderabad, India 12th International Symposium on Virus Diseases of Ornamental Plants, Haarlem, The Netherlands International viroid meeting, Berlin, Germany 3rd International Symposium on Legume and Vegetable Viruses in Ljubljana, Slovenia EUPRESO-DEP starting meeting, Aarhus, Denmark Joint meeting Dutch and German virologists, Hamburg, Germany 21st International Symposium on Fruit Tree Viruses in Neustadt, Germany EUPRESO-DEP concluding meeting, Ljubljana, Slovenia EPPO experts meeting on tomato viroids, Ljubljana, Slovenia COST Action FAO807 meeting, Sitges, Spain EFSA project Prima Phace kick-off meeting, Parma, Italy ▶ Presentations (from 2007) Situation of apple proliferation phytoplasma and pear decline phytoplasma in the Netherlands (oral) Pospiviroids in ornamental plants, and their potential risks for vegetable crops (oral) Pospiviroids in ornamental plants, and their potential risks of spread (oral) <i>Potato spindle tuber viroid</i>: sources of infection for tomato (oral) <i>Pepper chat fruit viroid</i>: biological and molecular characterization of a new viroid species from <i>Capsicum annuum</i> (oral) First report of <i>Tomato yellow leaf curl virus</i> in tomato in the Netherlands (poster) Pospiviroiden in bloemisterij- en groentegewassen (oral) Introduction to pospiviroids, and initiatives towards the detection of PSTVd and TCDVd in tomato seed (oral) <i>Pepper chat fruit viroid</i>: biological and molecular characterization of a new viroid species from <i>Capsicum annuum</i> (oral) Epidemiological evidence that vegetatively-propagated solanaceous plant species act as sources of <i>Potato spindle tuber viroid</i> inoculum for tomato (oral) Detection of TCDVd and PSTVd in seeds of tomato (poster) Surveys for new pospiviroid host plants (oral) Host-pospiviroid interactions (oral) Mechanical transmission of PSTVd (oral) Pospiviroid infections in tomato (oral) Impact and control of pospiviroids (oral) ▶ IAB interview ▶ Excursions 	<div style="text-align: right;"><i>date</i></div> <div style="text-align: right;">Apr 19-20, 2010</div> <div style="text-align: right;">Mar 2008</div> <div style="text-align: right;">Oct 2008</div> <div style="text-align: right;">May 2009</div> <div style="text-align: right;">Nov 2009</div> <div style="text-align: right;">Dec 2009</div> <div style="text-align: right;">Feb 27-28, 2007</div> <div style="text-align: right;">Oct 15-19, 2007</div> <div style="text-align: right;">Apr 20-24, 2008</div> <div style="text-align: right;">Jul 26-27 2008</div> <div style="text-align: right;">Aug 20-23, 2008</div> <div style="text-align: right;">Nov 04-05, 2008</div> <div style="text-align: right;">Apr 08-09, 2009</div> <div style="text-align: right;">Jul 05-10, 2009</div> <div style="text-align: right;">Oct 26-27 2009</div> <div style="text-align: right;">Oct 28-29 2009</div> <div style="text-align: right;">Jan 31-Feb 02, 2010</div> <div style="text-align: right;">Mar 09-11, 2010</div> <div style="text-align: right;">Feb 2007</div> <div style="text-align: right;">Oct 2007</div> <div style="text-align: right;">Apr 2008</div> <div style="text-align: right;">Jul 2008</div> <div style="text-align: right;">Aug 2008</div> <div style="text-align: right;">Aug 2008</div> <div style="text-align: right;">Oct 2008</div> <div style="text-align: right;">Feb 2009</div> <div style="text-align: right;">Apr 2009</div> <div style="text-align: right;">May 2009</div> <div style="text-align: right;">Aug 2009</div> <div style="text-align: right;">Oct 2009</div> <div style="text-align: right;">Oct 2009</div> <div style="text-align: right;">Oct 2009</div> <div style="text-align: right;">Oct 2009</div> <div style="text-align: right;">Oct 2009</div>
<i>Subtotal Scientific Exposure</i>	
24.3 credits*	
3) In-Depth Studies <ul style="list-style-type: none"> ▶ EPS courses or other PhD courses Course on phylogeny of viruses/viroids by dr R. Kormelink, WUR NL (March 26, April 10 and 19) Training in preparing viroid RNA sec. structures using mFold, RnaViz and Powerpoint by dr. M. de la Peña, Valencia, Spain ▶ Journal club Compiling of the Annual Newsletter International Working Group on Legume and Vegetable Viruses ▶ Individual research training 	<div style="text-align: right;"><i>date</i></div> <div style="text-align: right;">Mar 26, Apr 10, 19, 2006</div> <div style="text-align: right;">Oct 01-3, 2007</div> <div style="text-align: right;">2006-2008</div>
<i>Subtotal In-Depth Studies</i>	
3.8 credits*	
4) Personal development <ul style="list-style-type: none"> ▶ Skill training courses Academic writing Scientific writing ▶ Organisation of PhD students day, course or conference Co-organiser 12th International Symposium on Virus Diseases of Ornamental Plants, Haarlem, The Netherlands ▶ Membership of Board, Committee or PhD council Chairman of the KNPV Commission 'Nederlandse namen van plantenziekten' Secretary of the NKP Commission on Nomenclature Member Steering Committee International Working Group on Legume and Vegetable Viruses (secretary 2005-2008) Work Package Leader EUPHRESO-project Detection and epidemiology of pospiviroids (DEP) 	<div style="text-align: right;"><i>date</i></div> <div style="text-align: right;">Jan-Apr 2007</div> <div style="text-align: right;">Jan-Mar 2008</div> <div style="text-align: right;">Apr 2008</div> <div style="text-align: right;">2006-2010</div> <div style="text-align: right;">1997-2010</div> <div style="text-align: right;">2002-2009</div> <div style="text-align: right;">2008-2009</div>
<i>Subtotal Personal Development</i>	
5.7 credits*	
TOTAL NUMBER OF CREDIT POINTS*	
39,8	

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

* A credit represents a normative study load of 28 hours of study

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