Acta Biochimica Polonica

Vol. 48 No. 2/2001

467–476

QUARTERLY

Genetic variability of potato spindle tuber viroid RNA replicon★✪

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Received: 14 February, 2001; revised: 24 May, 2001; accepted: 31 May, 2001

Key words: infectious clones, pathogenicity, genetic stability, sequence diversity, potato spindle tuber viroid

The genetic continuity of the potato spindle tuber viroid (PSTVd) genome was analysed after infection of tomato plants with cloned cDNAs of parental strains. During the six weeks of the experiment, several new sequence variants appeared. The sequence variants detected in the progeny population induced sequence-specific disease symptoms. The PSTVd genome therefore follows the pattern expected for typical pseudo-strains propagating in plants as a population of similar sequences. Assessing further the replicon continuity, a PSTVd cDNA mutant with a deletion in the central conserved region was constructed and proven to be non-infectious. Surprisingly, in a sub-population of potato transformants expressing the same deleted PSTVd RNA an infectious viroid was detected. This suggests specific transcript conversion followed by recovery of the full-length pathogen genome.

The potato spindle tuber disease was the first of all viroid-induced diseases to be recognised and studied by plant pathologists. During the efforts to purify a putative infectious agent from potato spindle tuber-affected plants the unusual properties of the pathogen were recognised, the first viroid isolated, and the viroid concept developed. The term "viroid" was proposed (Diener, 1971) in order to differentiate these small, protein-free infectious RNAs from conventional viruses with an encapsidated genome. To date, over 20 differ-

★Presented at the International Conference on "Molecular Architecture of Evolution, Primary and Secondary Determinants" Poznañ, Poland, October 29–31, 2000.

✪This work was partly supported by the Polish-French Plant Biotechnology Centre (CNRS-KBN), and the State Committee for Scientific Research (KBN, Poland) grant No. P06A 02108.

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Abbreviations: CaMV, cauliflower mosaic virus; CCR, central conserved region; PSTVd, potato spindle tuber viroid.

ent viroids have been detected, the majority causing diseases of economically important crop plants.

The symptoms of potato spindle tuber viroid disease may vary considerably depending on the PSTVd strain, potato cultivar and environmental conditions. Typical foliar symptoms include stunting, uprightness, and small leaflets. Tubers are smaller, fewer in number, elongated, with numerous shallow eyes, and have abnormal skin colour and texture (Pfannenstiel & Slack, 1980; Chrzanowska *et al*., 1984; Kowalska-Noordam & Skrzeczkowska, 1984; Kowalska-Noordam *et al*., 1986/87).

As a standard, Rutgers tomato plants are used as test plants to propagate PSTVd and determine the symptom severity. According to the symptoms induced, the PSTVd strains are classified as mild, intermediate, severe, and lethal.

The first viroid sequence to be determined was that of the PSTVd intermediate strain (PSTVd-DI) (Gross *et al*., 1978). This single-stranded RNA consists of 359 nucleotides, and is circular. A unique rod-like structure, with a serial arrangement of double-helical sections and small internal loops was proposed. Taking into account all reported data, it was concluded that viroid RNAs are not translated into viroid-specific polypeptides (Zaitlin & Hariharasubramanian, 1972; Conejero & Semancik, 1977; Gross *et al*., 1978; Conejero *et al*., 1979; Camacho Henriquez & Sänger, 1982a; 1982b).

Comparative sequence analysis of different PSTVd family members has indicated the presence of five structural domains, designated: TL, P, C, V and TR (TL – left terminal, P – pathogenicity, C – central, V – variable, TR – right terminal), each responsible for different functions (Keese & Symons, 1985; 1987). The central conserved region (CCR) of the C domain may represent an important control region in viroid replication, potentially assuming alternative secondary structures in different replication steps (Keese & Symons, 1985; Diener, 1986; Sänger, 1987, Steger *et al*., 1992; Baumstark & Riesner, 1997).

Since the determination in 1978 of the first complete nucleotide sequence of the PSTVd intermediate strain (PSTVd-DI), the sequence of about forty different PSTVd sequence variants has been determined. Sequence analyses revealed that they differ from PSTVd-DI by only a few nucleotide changes such as substitutions, insertions, and deletions. The RNA chain length varies from 356 to 360 nucleotides. The mutations are mostly located in the P and V domains (Kuo, 1979; Gross *et al.,* 1981; Van Wezenbek *et al*., 1982; Schnölzer *et al*., 1985; Herold *et al*., 1992; Owens *et al*., 1992; Lakshman & Tavantzis, 1993; Góra *et al*., 1994; 1997). Sequence heterogeneity has been observed in natural viroid isolates. Such observations indicate that PSTVd, like many other RNA pathogens, propagates in the host as a population of similar but non-identical sequences comprising quasi-species. The quasi-species concept developed by Eigen and co-workers describes the complex behaviour of such populations acting as a whole (Eigen & Winkler-Oswatitsch, 1990; Eigen, 1993). To understand better the behaviour of quasi-species, as well as to describe it on mathematical foundations, the sequence space concept was introduced. In the space of given sequences, each possible sequence variant is represented as a single point. The points are ordered to form discrete, cellular and multi-dimensional space volumes reflecting the informational relationships between the sequences they represent. To each point in the sequence space a sequence fitness value can be assigned, resulting in a fitness landscape containing peaks and ridges of high fitness separated by saddles and valleys of lower fitness. Due to natural selection, higher positions of the fitness landscape become more populated than others. New mutants resulting from replication of sequences with a high fitness appear close to their parental sequences, thus also in the high fitness regions. This means that the

cloud of points representing the quasi-species as a whole is condensed by natural selection on the high fitness peaks and ridges. Thus, replication error rate and the shape of the fitness landscape are the two factors that finally determine the behaviour of the quasi-species in the sequence space. Such understanding of the relationships between individual mutants leads to the conclusion that the RNA genome may oscillate between several viable sequence versions of the replicon. If this is true, the specific PSTVd sequences detected while sequencing a population represent in fact transitory entities being momentarily preferred by conditions of the experiment, host physiology, or selective pressures created by the observer seeking specific phenotypes. To prove this hypothesis massive cloning and sequencing of the progeny of defined parental PSTVd strains were performed. Test plants were infected with preparations of cDNAs carrying the appropriate PSTVd sequences. Homogeneity of the parental cDNAs was confirmed by direct DNA sequencing. The variations observed in progeny sequences could point to the quasi-species nature of the PSTVd replicon.

MATERIALS AND METHODS

Viroid isolates and sequence variants nomenclature. The PSTVd isolates and sequence variants have been reported previously (Góra *et al.,* 1994; 1997). Three isolates of PSTVd: mild, intermediate and severe had been kept in the collection by consecutive passages in Rutgers tomato plants grown in greenhouses.

cDNA synthesis and cloning. Purified viroid RNAs extracted from PSTVd infected tomato leaves were reverse transcribed and the resulting cDNAs PCR amplified. Two specific primers corresponding to the CCR were used (Góra *et al*., 1994). To generate full-length monomeric infectious clones the cDNAs were ligated into the *Sma*I site of the pUC9 vector. The cloned PSTVd cDNAs were sequenced using a fluorescent primer sequencing kit and an automated A.L.F. sequencer (Pharmacia).

Infectivity assays. Tomato seedlings (cv. Rutgers) were inoculated with plasmids (2 μg/plant) containing a monomeric full-length cDNA of the appropriate sequence variant according to Cress *et al.* (1983) and Candresse *et al*. (1990).

Cloning of PSTVd cDNA into a binary vector. The *Sma*I-*Bam*HI restriction fragment of the S23 PSTVd cDNA was ligated into *Hin*cII cleaved pUC1813 vector (Kay & McPherson, 1987). This step was performed to add symmetric *Hin*dIII sites on both sides of the cDNA. The resulting *Hin*dIII fragment was re-cloned into the *Hin*dIII site of the binary pKYLX71-35S^2 vector (Maiti *et al*., 1993) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The plant expression cassette of the pKYLX71-35S^2 carries an enhanced 35S promoter of CaMV and the termination signal of the small subunit of the pea ribulose bisphosphate carboxylase gene (3′ rbcS). The resulting constructs with PSTVd cDNA (356 bp) in (+) and (–) orientation were used to transform *Agrobacterium tumefaciens* LBA 4404.

Potato transformation. Transformation of *Solanum tuberosum* (cv. Irga) was carried out according to the method of Martini *et al.* (1993) with some modifications (Góra-Sochacka *et al.*, 2000).

RESULTS

Sequence heterogeneity in phenotypically established PSTVd isolates

PSTVd field strains isolated after several passages in test plants are generally assumed by phytopathologists to be pure strains. However, under rigorous analysis they may show signs of hereditary instability. Inoculation with PSTVd RNAs isolated from a defined pa-

rental plant may result, in progeny plants, in a collection of phenotypes scattered around the typical one. In standard phytopathological experiments on type variant propagation, such variability of phenotypes is generally overlooked, only sub-populations of plants with desired phenotypes being selected for further pathogen propagation. This induces a bias in the evaluation of the genetic stability and sequence homogeneity of PSTVd variants. It should be stressed that viroids isolated from field plants often represent rather heterogeneous populations. The intensification of symptoms during consecutive passages, the scattering of phenotypes in infected populations and the heterogeneity of natural isolates question the standard view on genetic continuity/stability of a defined PSTVd strain. In fact, the quasi-species concept by itself asks for a re-analysis of the genetic continuity of PSTVd strains. According to the simplest hypothesis (Fig. 1), high genetic stability of the PSTVd replicon would result in the homogeneity of progeny populations of PSTVd variants. Assuming a low genetic stability resulting from a higher error rate of RNA replication, the progeny RNA populations should be heterogeneous, with a significant portion of sequence variants distant from the parental one. This could even lead to altered phenotypes in the progeny population (Fig. 1).

To test the genetic stability of PSTVd we took advantage of reports showing infectivity of cloned viroid cDNA (Candresse *et al*., 1990; Cress *et al*., 1983; Owens *et al*., 1986). The starting RNA preparations were defined by phytopathologists as type isolates inducing either severe, intermediate or mild disease symptoms. Such preparations (Table 1, left column) are used as standard PSTVd isolates in biological tests for viroid detection (Diener, 1987). Purified PSTVd RNA from these isolates was reverse transcribed and the resulting cDNAs were enzymatically amplified. The full-length PSTVd cDNAs obtained were cloned in the pUC9 vector. In this manner, a number of sequentially homogeneous, infectious PSTVd cDNA clones were obtained. These cDNA clones were sequenced and assayed for pathogenicity. The results are shown in Table 1. The severe and intermediate isolates were a mixture containing a few

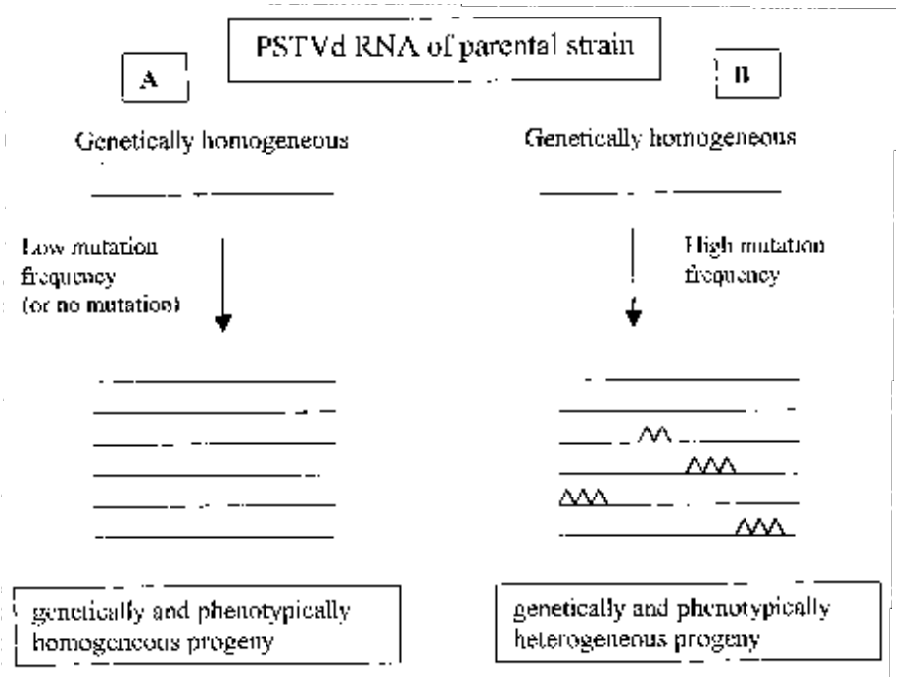

Figure 1. Hypothetical results of test plant infection with homogeneous PSTVd strain.

Δ: schematises a mutation.

sequence variants. In the mild isolate only one type of sequence was detected. The pathogenicity of each individual sequence variant was assayed. As shown in Fig. 2 and Table 1 the detected sequence variants induced different disease symptoms – from mild to severe. Surprisingly, divergence in symptom severity was observed even among the sequence variants present in the same RNA isolate defined by phytopathologists as the standard strain (Table 1).

Table 1. Sequence variants detected in PSTVd isolates.

RNA phytopathological standards were obtained from PSTVd strain collection. RNAs representing severe, intermediate and mild isolates were reverse transcribed and PCR-amplified (see Materials and Methods). The resulting PSTVd cDNAs were then cloned in the pUC9 vector. A collection of cDNA clones derived from each standard isolate was sequenced. Each molecular variant was separately tested for infectivity and phytopathological phenotype (see Material and Methods). PSTVd-I2 is identical to the previously described PSTVd-DI (Gross *et al.*, 1978). Frequency of a given sequence variant is expressed as the number of cDNA clones with the detected sequence per number of sequenced clones.

RNA standard PSTVd isolate with reported phenotype	Name and EMBL accession number of variants detected in analysed standard isolate	Phenotype induced by detected variant	Frequency of detected variant
Severe	PSTVd-S23 (X76846)	Severe	1/11
	PSTVd-S27 (X76845)	Severe	1/11
	PSTVd-I2 (V01465)	Intermediate	8/11
	PSTVd-I4 (X76848)	Intermediate	1/11
Intermediate	PSTVd-I2 (V01465)	Intermediate	8/10
	PSTVd-I3 (X76847)	Intermediate	1/10
	PSTVd-I4 (X76848)	Intermediate	1/10
Mild	PSTVd-M (X76844)	Mild	8/8

Hereditary phenotype fluctuation in PSTVd

Cloned PSTVd sequence variants as a rule induce well-defined disease symptoms in the majority of primary infected plants. However, the primary infection with PSTVd variant S27 was followed by evident disease phenotype instability. Only two of the ten inoculated plants developed the expected severe symptoms, whereas eight were symptomless. Looking further into phenotype heredity, the progeny population of S27 was isolated from symptomless hosts and inoculated to a next series of plants. Infected plants were allowed to grow for 6 weeks and the procedure of progeny isolation, cloning, sequencing and infection of the next generation of plants was repeated 5 times. The progeny PSTVd populations were analysed at the sequence level after the first and the sixth plant passage. Taking advantage of the cDNA cloning procedure, which makes it possible to obtain infectious clones, a specific phenotype was assigned to each sequence variant detected. The sequences of S27 progeny genomes were aligned. Figure 3 shows the graph of the sequence variants ordered in such a way that the sequences, which are neighbours in the graph, differ by a single point mutation (indicated to the right in the graph). Representatives of this series show surprisingly disparate phenotypes. It seems that the parental variant S27 (inducing severe symptoms) can be easily converted by a point mutation to the S27-I-8 variant, which induces mild symptoms. S27-I-8 itself can be converted to a severe mutant (S27-VI-106) by another additional point mutation. Next, this last variant can be further point mutated to yield another mild variant (S27-VI-19). Such rather unusual

Figure 2. Symp toms in duced by in di vid ual PSTVd se quence vari ants in Rutgers to mato plants one month after in oc u lation.

Seed lings were in oc u lated with plasmids (2 μg/plant) con tain ing a monomeric cDNA copy of the ap pro pri ate se quence vari ant. Z: healthy, mock in oc u lated plant; i2: PSTVd-I2, i3: PSTVd-I3, i4: PSTVd-I4 – s e quence vari ants in duc ing in ter me di ate dis ease symp toms. s23: PSTVd-S23, s27: PSTVd-S27 – se quence vari ants in duc ing se vere dis ease symp toms. M: PSTVd-M – se quence vari ant in duc ing mild dis ease symp toms. PSTVd-I2 is iden ti cal to the pre vi ously de scribed PSTVd-DI (Gross *et al.*, 1978).

fluctuations could be responsible for the disparate disease phenotypes frequently observed in populations of the primary infected host.

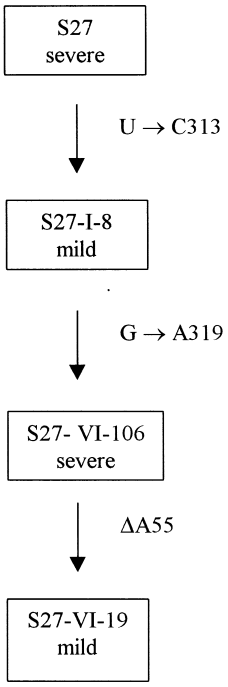

Figure 3. Proposed "family tree" of mutations in the S27 PSTVd family.

S27 – parental sequence (detected in the severe PSTVd isolate, see Table 1); S27-I-8 – sequence variant detected among progeny of S27 parental sequence after the first plant passage; S27-VI-106 and S27-VI-19 – sequence variants detected after the sixth plant passage. All nucleotide changes refer to differences with respect to the S27 sequence.

Recovery of infective viroid molecules from a truncated PSTVd transcript

The progeny of different PSTVd variants included a subset of viable genomes carrying short deletions. The viability of specific deletants was also reported in the literature (Wassenegger *et al*., 1994). In such cases, deletions present in the parental genotype were also detected in the progeny genomes. It therefore appears that short deletions could be considered as well-conserved in PSTVd. Expecting conservation of deletions, we decided to construct a non-infective PSTVd deletant truncated in the central conserved region believed to be crucial for PSTVd replication. The cDNA of the S23 PSTVd sequence variant with a two-nucleotide deletion ($C_{93}C_{94}$) in the central conserved region was cloned into the binary pKYLX71-35S2 vector under control of the CaMV 35S promoter. Constructs with the cDNA inserted in (+) and (–) orientations were obtained and used in *Agrobacterium*-mediated transformation of *Solanum tuberosum* (cv. Irga) leaf discs. This was expected to result in transgene-driven expression of the truncated, non-replicating and therefore non-infectious PSTVd genome. Such a truncated, non-viable PSTVd molecule could be of interest for studies on plant resistance to viroids.

A total of 113 transgenic lines were regenerated and grown *in vitro*. PCR analysis of the plant DNA confirmed the presence of the introduced constructs. Fifty selected transgenic plants were tested to evaluate their degree of resistance to PSTVd infection. Preliminary results indicate that none of them was substantially resistant to PSTVd. However, biological assays led to an unexpected observation. A few transgenic plants carrying the PSTVd cDNA construct in the (+) orientation (with expected (+) RNA expression) showed distinctive morphological changes – growth stunting and leaf malformation – similar to the disease symptoms caused by PSTVd. No such symptoms were observed in transgenic plants carrying the PSTVd cDNA in the (–) orientation. Sequence analysis revealed that these transformants accumulated infectious full-length viroid molecules.

DISCUSSION

Ab initio progeny analysis with the cloned parental standards led to rather unexpected observations on PSTVd variant phenotype heredity. The infective cDNA clones represent by definition populations of identical sequence. Homogeneity of the cDNA clones was verified by direct cDNA sequencing. Accord-

ing to the standard understanding (Fig. 1A) infection with such clones should lead to progenies identical in sequence and function to the parental one. This is evidently not the case as illustrated with the experiments done with the S27 sequence. After a single plant passage, the PSTVd progeny was already heterogeneous, with the heterogeneity increasing with each consecutive plant passage. Therefore, the hypothesis presented in Fig. 1B was confirmed. As the cloning procedure makes it possible to obtain infectious PSTVd cDNA clones, the disease phenotype induced by each sequence variant could be tested. This allowed us to pinpoint among the progeny sequences those which differ only by a single point mutation or deletion; surprisingly these mutants sometimes differed heavily in disease phenotype (Fig. 3). This means that starting from the master S27 sequence, the phenotype fluctuates in concert with point mutations, the first mutation converting the phenotype to mild, the second resulting in a severe phenotype, the third re-inducing mild symptoms. The mutations in question are located in the PSTVd P domain and formally can be compared to intragenic phenotype suppressors. One could expect attenuation followed by mild disease phenotype to be beneficial to pathogen propagation. In evolutionary timescales, the mild version should be preferred over the severe one, deleterious to the host. In fact, the mild sequence can re-create the severe sequence and oscillation of the phenotype (mild ↔ severe) is observed in the S27 family. With appropriate reservations, it seems that the disease phenotype induced is of importance to plant growers while probably being neutral to the pathogen. Taking into account that mild infection in a 6-week-old tomato plant gives around 6×10^{13} PSTVd molecules, and assuming that the severe disease diminishes the plant weight three fold, the severe genome still replicates in numbers assuring its successful propagation. It seems that the mutational rate in PSTVd 'probing' the sequence space is high enough to create, even during the time of the experiment, evolutionary fluctuations between parasitism and commensalisms. Therefore, selective pressure towards the elimination of the severe phenotype is periodically alleviated by the appearance of PSTVd versions propagating without damage or with reduced damage to the host. In general, such a "fluctuating" phenotype could be conceived as a new mechanism of chronic disease. Indeed it is a warning to phytopathologists. The mild infection — undetectable in the field — can lead to local severe disease foci appearing without contact with severely diseased plants during the growth season. This also means that infection with the mild variant cannot be considered a barrier for disease spread, since the mild variant by itself could be the source of severe mutants.

After identification of the effects of base substitutions, we focused our attention on the genetic continuity of specific deletions in the PSTVd replicon. In progeny population of parental genotypes, a few one- and two-nucleotide deletions in the P or the TL domain were detected (data not shown). Such deletions were followed by strong reduction in infectivity. As expected, deletions were not detected in the region believed to be crucial for viroid replication. Indeed, the recombinant plasmid carrying the PSTVd cDNA with a two base deletion in the CCR (see Methods) was not infectious (results in preparation). This strongly suggests that the effect of deletions in this region would be lethal. Therefore, we assume that expression of a transgene carrying this deletion in transgenic plants would lead to the appearance of a truncated PSTVd transcript unable to propagate in the host. However, contrary to expectations, in a sub-population of transgenic plants infectious full-length PSTVd molecules were observed. Direct sequencing of transgene confirmed the presence of the introduced deletion at the transgene level (to be published). At the present stage we assume that massive PSTVd-like transcript production in the transgenic plants may lead to occasional transcription of full-

length PSTVd molecules due to misincorporations by RNA polymerase. Even if such events are rare, they would be detected in the system we used, due to the ensuing amplification of any PSTVd mutant transcript having recovered the capacity to replicate. One can speculate that information lost at the DNA level under rather specific conditions could thus be retrieved at the RNA level.

REFERENCES

Baumstark, T., Schröder, A.R.W. & Riesner, D. (1997) Viroid processing: Switch from cleavage to ligation is driven by a change from a tetraloop to a loop E conformation. *EMBO J.* **16,** 599–610.

Camacho Henriquez, A. & Sänger, H.L. (1982a) Gel electrophoretic analysis of phenol-extractable leaf proteins from different viroid/host combinations. *Arch. Virol.* **74,** 167–180.

Camacho Henriquez, A. & Sänger, H.L. (1982b) Analysis of acid-extractable tomato leaf proteins after infection with a viroid, two viruses and a fungus and partial purification of the "pathogenesis-related" protein p14. *Arch. Virol.* **74,** 181–196.

Candresse, T., Diener, T.O. & Owens, R.A. (1990) The role of the viroid central conserved region in cDNA infectivity. *Virology* **175**, 232–237.

Chrzanowska, M., Kowalska-Noordam, A., Zagórska, H. & Skrzeczkowska, S. (1984) Reakcja polskich odmian ziemniaka na silny szczep wiroida wrzecionowatości bulw. *Biuletyn Instytutu Ziemniaka* **31,** 15–27 (in Polish).

Conejero, V., Picazo, I. & Segado, P. (1979) Citrus exocortis viroid (CEV): Protein alternations in different hosts following viroid infection. *Virology* **97,** 454–456.

Conejero, V. & Semancik, J.S. (1977) Exocortis viroid: Alternation in the proteins of *Gynura aurantiaca* accompanying viroid infection. *Virology* **77**, 221–232.

Cress, D.E., Kiefer, M.C. & Owens, R.A. (1983) Construction of infectious potato spindle tuber viroid cDNA clones. *Nucleic Acids Res.* **11,** 6821–6835.

Diener, T.O. (1971) Potato spindle tuber "virus". IV. A replicating, low molecular weight RNA. *Virology* **45**, 411–428.

Diener, T.O. (1986) Viroid processing: A model involving the central conserved region and hairpin I. *Proc. Natl. Acad. Sci. U.S.A.* **83,** 58–62.

Diener, T.O. (1987) Potato spindle tuber (diagnosis); in *The Viroids* (Diener, T.O., ed.) pp. 228–230, Plenum Press, New York.

Eigen, M. (1993) The origin of genetic information: Viruses as models. *Gene* **135,** 37–47.

Eigen, M. & Winkler-Oswatitsch, R. (1990) Statistical geometry on sequence space. *Methods Enzymol.* **183,** 505–530.

Góra, A., Candresse, T. & Zagórski, W. (1994) Analysis of the population structure of three phenotypically different PSTVd isolates. *Arch. Virol.* **138,** 233–245.

Góra-Sochacka, A., Kierzek, A., Candresse, T. & Zagórski, W. (1997) The genetic stability of potato spindle tuber viroid (PSTVd) molecular variants. *RNA* **3**, 68–74.

Góra-Sochacka, A., Rosa, I., Podstolski, W., Candresse, T. & Zagórski, W. (2000) Transgenic potato plants carrying truncated cDNA copy of a severe strain of PSTVd; in *Use of Agriculturally Important Genes in Biotechnology, NATO Science Series A, Life Sciences,* IOS Press, **319,** 14–18.

Gross, H.J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H. & Sänger, H.L. (1978) Nucleotidesequence and secondarystructure of potato spindle tuber viroid. *Nature* **273,** 203–208.

Gross, H.J., Liebel, U., Alberty, H., Krupp, G., Domdey, H., Ramm, K. & Sänger, H.L. (1981) A severe and mild potato spindle tuber viroid isolate differ in three nucleotide exchanges only. *Biosci. Rep.* **1,** 235–241.

Herold, T., Hass, B., Singh, R.P., Boucher, A. & Sänger, H.L. (1992) Sequence analysis of five new field isolates demonstrates that the chain length of potato spindle tuber viroid (PSTVd)

is not strictly conserved but as variable as in other vir oids. *Plant Mol. Biol.* **19,** 329–333.

Kay, R. & McPherson, J. (1987) Hybrid pUC vectors for addition of new restriction enzyme sites to the ends of DNA frag ments. *Nucleic Acids Res.* **15**, 2778.

Keese, P. & Symons, R.H. (1985) Do mains in viroids: Evidence of intermolecular RNA rearrangements and their contribution to viroid evolution. *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4582–4586.

Keese, P. & Symons, R.H. (1987) The struc ture of vir oids and virusoids; in *Vir oids and Viroid-like Pathogens* (Semancik, J.S., ed.) pp. 1–47, CRC Press, Boca Raton, FL.

Kowalska-Noordam, A. & Skrzeczkowska, S. (1984) Reakcja roœlin ziemniaka rodu PW 22/70 na zaka¿enie silnym i s³abym szczepem PSTV. *Biuletyn Instytutu Ziemniaka* **31**, 29–43 (in Pol ish).

Kowalska-Noordam, A., Chrzanowska, M. & Skrzeczkowska, S. (1986/87) Reaction of ten Polish potato cultivars to severe and mild strains of po tato spin dle tu ber viroid. *The Potato,* 71–91.

Kuo, T.T. (1979) Nu cle o tide se quence and sec ondary structure of potato spindle tuber viroid. *NTU Phytopathol. Enzymol.* **6**, 1–9.

Lakshman, D.K. & Tavantzis, S.M. (1993) Pri mary and sec ond ary struc ture of 360-nucleotide isolate of po tato spin dle tu ber viroid. *Arch. Virol.* **128**, 319–331.

Maiti, I.B., Murthy, J.F., Shaw, J.G. & Hunt, A.G. (1993) Plants that express a potyvirus proteinase gene are resistant to virus infection. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6110–6114.

Mar tini, N., Egen, M., Runtz, I. & Strittmatter, G. (1993) Promoter sequence of potato pathogenesis-related gene mediate transcriptional activation upon fungal in fec tion. *Mol. Gen. Genet.* **236**, 179–186.

Owens, R.A., Hammond, R.W., Gardner, R.C., Kiefer, M.C., Thompson, S. & Cress, D.E. (1986) Site specific mutagenesis of potato spin dle tu ber viroid cDNA. *Plant Mol. Biol.* **6**, 179–192.

Owens, R.A., Khurana, S.M., Smith, D.R., Singh, M.N. & Garg, I.D. (1992) A new mild strain of po tato spin dle tu ber viroid iso lated from wild *Solanum* spp. in In dia. *Plant Dis.* **76,** 527–529.

Pfannenstiel, M.A. & Slack, S.A. (1980) Re sponse of potato cultivars to infection by the potato spindle tuber viroid. *Phytopathology* **70,** 922–926.

Sänger, H.L. (1987) Viroid func tion – viroid rep lication; in *The Viroids* (Diener, T.O., ed.,) pp. 63–98, Ple num, New York.

Schnölzer, M., Hass, B., Ramm, K., Hofmann, H. & Sänger, H.L. (1985) Correlation between struc ture and patho ge nic ity of po tato spin dle tu ber viroid (PSTVd). *EMBO J.* **4,** 2181–2190.

Steger, G., Baumstark, T., Morchen, M., Tabler, M., Tsagris, M., Sänger, H.L. & Riesner, D. (1992) Struc tural re quire ments for viroid processing by RNase T1. *J. Mol. Biol.* **227,** 719–737.

Van Wezenbek, P., Vos, P., Van Boom, J. & Van Kammen, A. (1982) Molecular cloning and characterization of a complete DNA copy of po tato spin dle tu ber viroid RNA. *Nucleic Acids Res.* **10,** 7947–7957.

Wassenegger, M., Heimes, S. & Sänger, H.L. (1994) An infectious viroid RNA replicon evolved from an *in vitro*-generated non-infectious viroid deletion mutant via a comple mentary deletion *in vivo*. *EMBO J.* **13,** 6172–6177.

Zaitlin, M. & Hariharasubramanian, V. (1972) A gel electrophoretic analysis of proteins from plants infected with tobacco mosaic and potato tu ber vi ruses. *Virology* **47,** 296–305.