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VIROIDS: MOLECULAR INFECTIOUS AGENTS

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Summary. — In 1971, unique small RNA molecules, the viroids, were found to cause specific infectious diseases of plants. They are the smallest and simplest contagious agents known. Until now, 14 viroids have been described and 12 diseases of potatoes, tomatoes, citruses, chrysanthemums, cucumbers, hops, coconut palms, avocado trees and burdock are known to be caused by viroids. The common symptoms of these diseases are: stunting of plants, discoloration of veins, epinasty, curling and distortions of leaves, chlorotic or necrotic spots etc., followed by death of the diseased plants. All viroids are ssRNAs of m.w. ranging from 1.1×10^5 to 1.7×10^5, corresponding to chains of just 246 to 371 ribonucleotides. For 10 viroids, complete nucleotide sequences are known. PSTV, CSV, CEV, TPMV and TASV show 60%—80% homology with each other; in analogy, ASBV, HSV, CPFV, GV and CCCV are closely homologous to each other, too, but just distantly related to the PSTV group. Extensive intramolecular base pairing creates a characteristic secondary structure of the cyclic viroid RNA chain, native viroids appearing as quasi double-stranded, unbranched, very short rod-like structures with short single-stranded loops. (Thus PSTV forms rods about 50 nm long and 2 nm wide.) The stretch of nearly all viroids bears a common central conserved region of 19 bp. The "upper" part of this region is, presumably, the cleavage-ligation site of viroid oligomers during replication. Viroids are located and replicated in nuclei of infected cells, in association with their nucleoli. Their replication is directed by host DNA-dependent RNA polymerase II using cRNA oligomers as templates according to the rolling circle model. Viroid RNA has no mRNA function. The virulence of viroids is coded by their virulence modulating region in the "left hand" part of their molecules; a single nucleotide substitution between nucleotides 43 and 56 within this region alters the virulence. Most probably, viroids have originated by the circularization of spliced-out transcripts of eucaryotic introns. A stable complex may be created between the 5' end of U1 snRNA and nucleotides 257 to 279 of PSTV cRNA strand; thus the pathogenic effects of viroids seem to be a result of their interference with pre-mRNA processing.

Key words: Viroids; virusoids; ssRNA; small RNA molecules; nucleotide boxes; hairpin structure; cRNA template; rolling circle model; DNA-dependent RNA polymerase; escaped introns; plant pathogens

Viruses — Viroids — Virusoids; a Short Historical View

The great microbiological discoveries of the 19th century showed a variety of cellular microorganisms to be causative agents of infectious diseases of plants, animals and humans; the smallest of them were microscopic fungi and bacteria. However ,during the last decade of the 19th century, the fundamental discoveries of Ivanovskij (1892), Beijerinck (1898) and of Loeffler and Frosch (1898) proved the existence of still much smaller infectious agents: of submicroscopic, subcellular particles — the viruses.

For 80 years following their discovery, viruses were believed to be the smallest and simplest contagious agents. Nevertheless, in 1971 a surprising, quite unexpected finding was reported by Diener (1971*a*), which has changed this general opinion: the finding of specific small RNA molecules acting as causative agents of plant infections, i.e. as plant cell parasites — the discovery of viroids. Pure RNA molecules can hardly be regarded as alive — but yet they are replicated by plant tissue cells, displaying their molecular genetic continuity, thus causing characteristic symptoms of their infection, just as viral genomes do.

And only 10 years later, in 1981, viroid-like RNA molecules were detected within virions of certain plant viruses, in addition to their own genomic RNAs (Randles *et al.*, 1981, Gould, 1981). The replication of these small RNAs is not driven autonomously, in contrast to viroids, but requires viral RNA molecules as helpers. They have been called virusoids (Jones, Mayo and Duncan, 1983).

Contemporary knowledge on viroids and virusoids has been summarized in special reviews and monographs; the most recent ones have been published by Diener (1981 *a*, *b*, 1982 *a*, *b*, 1983 *a*, *b*, 1984), Sänger (1982, 1984), Riesner *et al.* (1983), Maramorosch and McKelvey (1985), Šmarda (1985) and Riesner and Gross (1985).

The Discovery of Viroids

It had been assumed for years that the causative agent of the rather common infectious disease of potato (*Solanum tuberosum*), of spindle tuber disease, was a virus. The leading symptoms of this disease are spindle-like elongated, knot-deformed tubers with numerous surface fissures. Over the years several attempts were undertaken to isolate the virus, but without success.

An improved opportunity for the research on this infectious agent was established by Raymer and O'Brien (1962) who showed that it could be easily transferred from potato to young tomato plants; being readily replicated in leaves of *Lycopersicon peruvianum* just as of cultivars of *Lyco-*

Table 1. Molecular Weights of Some Viroids

Viroid	Abbreviation	Mol. weight	Standard deviation
potato spindle tuber viroid	PSTV	127,000	±4,000
citrus exocortis viroid	CEV	119,000	±4,000
cucumber pale fruit viroid	CPFV	110,000	±5,000

persicon esculentum, it caused characteristic symptoms during a considerably shorter time. However, attempts to isolate a virus from infected tomato leaf extracts failed again; it was impossible to spin the agent down even by ultracentrifugation at 100,000 × g for 4 hr. In sucrose density gradient it was deposited even more slowly than isolated viral nucleic acids. At the same time, it was inactivated by ribonuclease, but was not impaired by deoxyribonuclease or proteases. Thus the agent was shown to be a very short RNA molecule, not surrounded by a protein coat (Diener and Raymer, 1967). The size of this infectious RNA was then estimated by a combination of sedimentation and gel electrophoresis analyses. The infectious RNA was shown to be extremely small: its molecular weight (m.w.) being about 130,000. This contradicted sharply the axiome that the minimum size of independently replicating virus genomes must correspond to at least 1 million (Diener, 1971*b*).

The multiplication of such RNA molecules seemed first to be best explained as a replication of capside-less virus RNA depending on a helper virus. However, all attempts to find such a virus in tomato leaves failed. A capability of small RNA molecules to assemble into a viral genome was ruled out as well. It became clear that the small infectious RNA molecules themselves were replicated in host cells (Diener, 1971*a*, 1972).

The infective agent of potato spindle tuber disease appeared to constitute the first known representative of a new class of subcellular and subviral pathogens, for which the term viroid was introduced by Diener (1971*b*). Meanwhile, Sänger (1972) and, independently, Semancik and Weathers (1972) proved that the infectious exocortis of citrus trees was caused by a viroid as well. In 1973, Diener and Lawson established a further viroid to cause a severe ornamental disease: the chrysanthemum stunt. In addition, the subcellular agent of tomato bunchy-top (Benson *et al.*, 1965) was shown to be a viroid.

Nowadays, 14 viroids have been described and 12 plant diseases are known to be caused by viroids; viroids affect as diverse cultivated plant species as potatoes, tomatoes, citruses, chrysanthemums, cucumbers, hops, coconut palms and avocado trees, just as an uncultivated one — burdock (see below; for detailed survey see Sänger, 1984). There is little doubt that still more plant infectious diseases of — until now — unknown etiology will be identified later as being due to viroids.

Molecular Parameters of Viroids

All viroids are single-stranded ssRNAs of m.w. ranging from 1.1×10^5 to 1.7×10^5. The most precise estimations of viroid m.w. were achieved through

Table 2. RNA Chain Lengths of Some Viroids

Viroid	Abbreviation	Chain length (No. of nucleotides)	Reference
potato spindle tuber viroid	PSTV	359	Gross *et al.* (1978)
chrysanthemum stunt viroid	CSV	356	Haseloff and Symons (1981
citrus exocortis viroid	CEV	371	Visvader *et al.* (1982)
tomato planta macho viroid	TPMV	360	Kiefer, Owens and Diener (1983)
tomato apical stunt viroid	TASV	360	Kiefer, Owens and Diener (1983)
avocado sun blotch viroid	ASBV	247	Symons (1981)
hop stunt viroid	HSV	297	Ohno *et al.* (1983)
cucumber pale fruit viroid	CPFV	303	Sano *et al.* (1984)
grapevine viroid	GV	297	Sano *et al.* (1986)
coconut cadang-cadang viroid	CCCV	246	Haseloff, Mohamed and Symons (1982)

equilibrium ultracentrifugation under denaturing conditions (Sänger *et al.*, 1976). Examples of them are given in Table 1.

To these low m.w. of viroid RNAs correspond the low numbers of nucleotides (Table 2).

Viroid RNA chains are thus more than 20× shorter than RNA chains of the smallest viruses (RNA bacteriophages) known; e.g. ssRNA of phage f2 has the weight of 3.5×10^6 (Epps, 1981). In general, viroid RNAs are composed of about 240—370 nucleotides.

On the basis of sequence analyses and of results of further biochemical and biophysical studies, it has been established that viroids follow a common general principle of structure and replication (Riesner *et al.*, 1983).

As the first one, the complete nucleotide sequence of PSTV was established by Gross *et al.* (1978). PSTV sequence comprises 359 nucleotides: 73 adenosine-, 101 guanosine-, 108 cytidine- and 77 uridine monophosphates.

Since then, complete primary structures of nine further viroids have been established by nucleotide sequencing (compare Table 2). The surplus of cytidine- and guanosinephosphates seems to be generally characteristic of viroids. Also, an unusually long stretch of 18 purines (mostly adenines) found in positions 48—65 in PSTV, is probably common to all viroids.

Comparative sequence analyses have revealed two groups among viroids. PSTV, CSV, CEV, TPMV and TASV (about 360 nucleotides long) form the PSTV group, sharing 60—80% sequence homology with each other and cca 70—85% homology with PSTV (Sänger, 1982). On the other hand, considerably shorter sequences of ASBV, HSV, CPFV, GV and CCCV, sharing also close homolgy with each other, are only distantly related to PSTV group. Thus, PSTV shares 83% nucleotide sequence homology with TPMV, but only 55% homology with HSV; HSV, on the other hand, shares 95% homo-

logy with CPFV and GV. From sequence comparative studies it was concluded that CPFV is a cucumber isolate and GV a grapevine isolate of HSV (Sano *et al.*, 1984, 1986).

Under physiological conditions, extensive intramolecular base pairing creates a characteristic secondary structure of the linear viroid RNA chain; it is derived from a covalently closed single-strand (McClements and Kaesberg, 1977). Native viroids appear as double-stranded, covalently linked and hence unbranched, very short rod-like structures; in these structures, short double helices are spaced by short single-stranded loops (Sänger *et al.*, 1976).

In the molecule of PSTV, altogether 122 nucleotides are paired by hydrogen bonds to 122 complementary ones; in this way, altogether 26 double-stranded segments of 2—8 nucleotide pairs are formed and separated from each other by 25 single-stranded loops of non-complementary nucleotides; loops are supposed to be located also at either end of the viroid "rod". No modified nucleotide was found. Analogous secondary and tertiary structures have been proposed for other viroids of both groups, with the exception of ASBV and CCCV (Stenger *et al.*, 1984; Diener, 1986).

Among the structural features common to nearly all viroids, the most conspicious one is the "central conserved region". This region is represented by a stretch of 20 nucleotides in one strand and by a complementary sequence of 19 nucleotides (interrupted by three nonconserved ones) in the other one, situated directly "below" the first one; both stretches are located close to the centre of the "double-stranded" viroid molecule (Visvader *et al.*, 1982). The upper part of the central conserved region is, presumably, the cleavage-ligation site of viroid oligomers during replication.

Thermal denaturation studies have revealed that rod-like structures of viroids can be switched over to branched ones, marked by transient hairpin-like configurations (Riesner *et al.*, 1979). Two to three "secondary hairpins", not present in the native configuration, are usually signed I, II, III; they result from inverted repeats in viroid primary structure.

In an electron microscope, purified PSTV appears as very short rods, about 50 nm long and 2 nm wide (Sogo, Koller and Diener, 1973). The denatured, single-stranded PSTV molecule is 100 nm long (Riesner *et al.*, 1979). (Denaturing temperature T_m, sufficient for the denaturation of 50% molecules, in specific viroids amounts to 50—58 °C.)

Following a complete denaturation of PSTV, McClements and Kaesberg (1977) found three types of its molecules in electron microscope. In such preparations, about 10% molecules remained undenatured, 20% were circular, covalently closed single-stranded molecules of a mean contour length of 140 nm, and 70% were single-stranded linear molecules about 110 nm long.

Owens *et al.* (1977) separated circular molecules of PSTV from linear ones by gel electrophoresis. A test on tomato plants showed both molecule types to be infectious.

These details of the in vitro structure of viroids may be relevant to those in vivo, but there are no direct, reliable data on the actual structural state of viroids within the host cells available so far.

Molecular Specificity and Stability

Of the nucleotide sequence of CSV 69% can also be found in PSTV and both viroid RNAs can form analogous secondary structures (Haseloff and Symons, 1981). In spite of that, each viroid type (or species?) represents a molecular structure sui generis, specific of the given plant disease; this structure is more or less different from those of other viroids.

cDNA of PSTV RNA, replicated in potato plants, was synthesized in vitro (Owens, 1978); it hybridized with PSTVs replicated in *Chrysanthemum*, *Gynura* and *Lycopersicon*. In all cases, a complete hybridization was achived. In this way it was shown that the specific primary structure of PSTV is not altered by a host organism. Individual viroid types (PSTV, CEV, CSV) differ from each other outside the central conserved region.

On the other hand, several clones (or strains?) of PSTV are known, displaying minor differences of nucleotide sequence. Infection of potato or tomato plants by individual ones provokes the same disease symptoms in all cases, but differing in their severity; thus mild, medium and severe PSTV clones may be distinguished (Dickson *et al.*, 1979). Both a mild and a severe strain were sequenced and their primary structures compared. The mild strain was shown to differ from the severe one by just three nucleotide mutations, namely by the exchange of AA to U in positions 120—121, A to U in position 310 and by the insertion of U between positions 312 and 313 (Gross *et al.*, 1981).

In analogy, just tiny differences have been revealed by Gross *et al.* (1982) at primary and secondary structure comparison of various isolates of CSV (10 nucleotide exchanges) and of CEV (just 2 nucleotide exchanges).

It may be concluded that viroids constitute molecular genetic systems whose markers are encoded by the nucleotide sequences of their RNAs. These RNAs are subject to spontaneous point mutations resulting in tiny differences in their primary structure and, consequently, in a slight variation of their biological properties, but not influencing their type specificity. Flores (1984) postulated for all viroids of the PSTV group that their pathogenicity is associated with the conformation of the region between nucleotides 43—44 and 54—56, particularly at some specific points; this "region of mutations" is a part of the "virulence modulating (VM) region" (Schnölzer *et al.*, 1985 — see below).

Viroids in Host Cells

Bioassays of subcellular fractions from PSTV-infected tomato leaves showed that appreciable infectivity was bound (besides on debris) only to nuclear fractions (Diener, 1971*a*). Chloroplast, mitochondrial, ribosomal and cytoplasmic fractions contained only traces of infectivity. In cell nuclei, most infectivity was associated with chromatin, from which the infectious substance could be extracted with phosphate buffer as free RNA.

CEV is also primarily located in the nuclear fraction of *Citrus* cells and in close association with chromatin (Sänger, 1972), but in *Gynura aurantiaca* a significant portion of the viroid is associated with the endocellular biomembrane system (Semancik *et al.*, 1976).

Results obtained in an in vitro synthesizing system, where purified cell nuclei from infected tomato leaves were used as an enzyme source, supported the assumption that viroids are replicated in the nuclei. A comparative cytological analysis of infected and non-infected tomato leaf tissues revealed an enlargement of cell nuclei and enhanced RNA : DNA and protein : DNA ratios in the infected ones (Takahashi and Diener, 1975).

It appears, therefore, that the infecting viroid migrates into the nucleus to be replicated there; the mechanism of this motion is not yet known. Nearly all of the viroid progeny remains in the nucleus.

From further analyses it was concluded that viroids are associated with the nucleoli (Schumacher, Sänger and Riesner, 1983), most probably complexed via a protein-nucleic acid interac ion. In nucleoli, viroids were found predominantly in a nucleosomal fraction, bound in complexes of 12—15 S (characteristic of nucleosomes and their oligomers). In vitro reconstitution experiments showed viroids to enter covalently cross-linked complexes with proteins: with 4 histones and with two proteins of higher molecular weights: of 41,000 and, to a very small (perhaps insignificant) extent, with a protein of m.w. 31,000 (Wolff *et al.*, 1985). These proteins may be necessary to stabilize the tertiary structure of viroids (see below).

The binding of viroids to histones is in agreement with a certain DNA -similarity of viroids. This was first argued on the basis of their topological and thermodynamic properties (Riesner *et al.*, 1979). Owing to this similarity, viroids can serve as templates for the DNA-dependent RNA polymerase II (Rackwitz, Rohde and Sänger, 1981).

Viroid polynucleotide chains are of sufficient lenght to code for polypeptides of molecular weights about 10,000 (not taking into account the uneven number of nucleotides in the circular molecules — e.g. 359 in PSTV — which permits theoretically three rounds of translation following two successive frame shifts).

Because of this estimation, several authors tried to prove mRNA functions in viroids. However, tests for in vitro messenger function of PSTV and CEV in various cell-free protein-synthesizing systems indicated that neither of them functions in this way (Davies, Kaesberg and Diener, 1974; Hall *et al.*, 1974). Nor is CEV translated into peptide in *Xenopus laevis* oocytes, even following polyadenylation in vitro; it also does not interfere with the translation of genuine mRNAs (Semancik, Conejero and Gerhart, 1977).

Although viroids do not play the role of mRNAs, the theoretical possibility still remains that complementary RNA strands (cPSTV etc. — strands of viroids) could serve in this way. In fact, RNA sequences cemplementary to viroids are present in infected tissue cells (Grill and Semancik, 1978). Translation of cPSTV strands detected could result, theoretically, in the synthesis of four polypeptides (comprising 108, 79, 43 and 28 aminoacids, respectively — Matthews, 1978).

However, comparative analyses of proteins in healthy and in PSTV-infected tomato tissues (Zaitlin and Hariharasubramanian, 1972; Zelcer *et al.*, 1981), just as in healthy and CEV-infected *Gynura aurantiaca* (Conejero and

Semancik, 1977), did not reveal any qualitative differences. The only difference shown in both systems was an enhanced synthesis of two host cell-specific proteins (Conejero, Picazo and Segado, 1979).

In conclusion, in the light of present knowledge, neither viroids nor their cRNA strands do act as mRNAs. cRNA strands in viroid-infected cells must be synthesized solely by a preexisting (possibly activated) host RNA polymerase.

Replication of Viroids

An extensive research attention has been also paid to the mechanism of viroid replication: to the questions of template, of replication directing enzymatic machinery and to the course of molecular events during replication.

Theoretically, viroid replication could involve transcription from either RNA or DNA templates; and many contradictory and confusing data have been reported in favour of both possibilities.

However, the first results reporting the existence of viroid-complementary sequences in chromosomal DNA of plant cells have been proved erroneous. In exact experiments, no PSTV-complementary regions could be identified in nuclear DNA from both uninfected or viroid-infected tomato plants, using solubilization and hybridization techniques (Zaitlin *et al.*, 1980; Hadidi, Cress and Diener, 1981). The same result has been achieved also for CSV (Palukaitis, 1980). Nowadays it is conclusively evident that viroid replication proceeds via RNA templates.

The persuading argument was brought by Grill and Semancik (1978): whereas no viroid-related DNA requences could be identified in *Citrus* leaf tissue infected with CEV, CEV-complementary RNAs were present in their extracts. On the other hand, cCEV could not be found in healthy tissue (Grill, Negruk and Semancik, 1980). The same experience has been achieved by Owens and Cress (1980) for PSTV-infected and healthy tomato tissues. Most, if not all, cPSTV strands formed ribonuclease-resistant duplexes with PSTV RNA indicating a 100% sequence complementarity of both.

Also the observations that viroid sequence remains faithfully maintained during infection process, regardless of host species replicating viroid (Dickson, Diener and Robertson, 1978; Niblett *et al.*, 1978; Owens, Smith and Diener, 1978), support the idea of RNA templates for viroid reproduction.

Enzyme(s) Involved in Replication

Inhibition of replication in tomato leaves by 10^{-8} M α-amanitine (intracellular concentration) strongly suggested the specific involvement of DNA-directed RNA polymerase II in viroid transcription. This concentration is sufficient neither to inhibit RNA polymerase III, nor to affect the biosynthesis of tobacco mosaic virus RNA (Mühlbach and Sänger, 1979).

Rackwitz, Rohde and Sänger (1981), using blotting techniques, have shown that viroids enter readily into binary complexes with RNA polymerase II, the concentration of complexes formed being a function of RNA polymerase

II concentration. Both circular and linear PSTV molecules formed these complexes. Viroids compete with DNA for the template binding sites on this enzyme and thus strongly inhibit DNA-directed RNA synthesis.

Purified DNA-directed RNA polymerases II from wheat germs and from callus cells or leaves of wild tomato plants (*Lycopersicon peruvianum*) are equally capable of transcribing all viroids tested (Rackwitz, Rohde and Sänger, 1981). The efficiency of transcription of RNA templates by this enzyme is about one order of magnitude lower than of DNA ones, but viroids are transcribed with a higher efficiency than any other natural RNA template tested.

Rackwitz, Rohde and Sänger (1981) were also successful in molecular hybridization of full-length cPSTV strands, synthesized by this enzyme in vitro, with native PSTV circular and linear molecules.

There is no doubt that viroids can be replicated by a mechanism previously not known for replication of infectious RNA molecules: that they are copied by a pre-existing host cell enzyme whose standard cellular function is to transcribe DNA into pre-mRNA. It is tempting to speculate that it is the quasi double-stranded, DNA-like structure of native viroid molecules that introduces the DNA-directed RNA polymerase II molecules to a false template. In this view, viroids misuse an important cellular enzyme for their own replication and hence may be regarded — in analogy to selfish DNA molecules coding solely for their own sequence and maintenance — as selfish RNAs.

Nevertheless, it may be admitted that misuse of RNA polymerase II is not a necessary prerequisite for the reproduction of viroids. Below, the hypothesis of viroids as transcripts of escaped introns will be considered. If we accept this hypothesis as correct, we must admit that viroid molecules do nor originate by replication of previous ones, but that they are formed de novo: through splicing newly polymerized pre-mRNA strands. Furthermore, all persuading evidence on DNA-directed RNA polymerase activity in copying viroid RNA stems from experiments in vitro; no study has so far clearly identified any host enzyme copying it in vivo.

The recent experience on the location of viroid RNA in nucleoli (Schumacher, Sänger and Riesner, 1983), just as the discovery of sequences in viroids homologous to major portions of promotor sequence for a mouse rRNA gene (Palukaitis and Zaitlin, 1983) have suggested a possible involvement of a DNA-directed RNA polymerase I in viroid replication in vivo. This possibility was strengthened by the finding (Semancik and Harper, 1984) of traces of α-amanitineresistant CEV replication in vitro. Also RNA-directed RNA polymerase of plant cells might take the task of viroid transcription.

For covalent closing of linear molecules into circulat ones, RNA ligase activity must be anticipated. Such an activity has been proved e.g. in wheat germ (Branch *et al.*, 1982).

Molecular Mechanism of Reproduction, Replication Intermediates

Grill and Semancik (1978) were the first who showed that viroids are transcribed from RNA templates (see above). This finding was convincingly

supported by Owens and Cress (1980) in blot hybridization experiments using a PSTV-specific dsDNA, cloned in plasmid pBR322, as a probe. In both studies it could be shown that infected plant tissues contain linear RNA molecules complementary to viroids — (RNAs/cRNAs) of the same electrophoretic mobility under denaturing conditions, and hence of the same molecular weight, but of opposite polarity.

Soon thereafter, evidence showed that viroid-infected cells contain, in addition to full-length linear viroid RNA complements, viroid-specific complementary molecules longer than one unit length; they were identified as cRNA oligomers (Hadidi and Hashimoto, 1981; Rohde and Sänger, 1981). In Northern blot hybridization study of RNA in infected tissue extracts, Branch, Robertson and Dickson (1981) identified 4 discrete bands of cPSTV molecules under fully denaturing conditions: those of approximately 700, 1050, 1500 and 1800 nucleotides long, presumably representing polymers of PSTV, which — theoretically — should have contained 718 (dimer), 1077 (trimer), 1436 (tetramer) and 1795 (pentamer) nucleotides. No unit-length (359 nucleotides) cPSTV strands were detected, may be because of hybridization interference of unlabelled PSTV RNA moving in the gel together with cPSTV monomers. All cPSTV molecules were extracted as complexes with extensive double-strand regions, some of which being probably formed by unit-length dsRNAs flanked by single-strand sequences being about 40 nucleotides longer than unit-length. However, following a gentle treatment with some RNase under conditions favoring digestion of single-stranded regions, cPSTV oligomers could no longer be detected and roughly unit--length cPSTVs appeared. The authors concluded that the complexes found represent PSTV replication intermediates, whose (—)strands are formed by approximately viroid-length tandem repeats. They proposed that the template for cPSTV is the circular form of PSTV functioning according to the rolling circle RNA template model (Brown and Martin, 1965). They found a further support for this model in their observation that tomato plants infected with CEV contain cCEV strands whose estimated sizes were multiples of unit-length CEV.

In agreement with these findings and in support of the rolling circle model of viroid template function in its own replication, in RNA extracts from PSTV-infected tomato tissues viroid-related dsRNAs migrating more slowly in gels than unit-length single-stranded PSTV were found (Owens and Diener, 1982). By skilled separation analysis, replicative intermediates were proved in them containing monomeric circular or linear PSTV strands complexed with oligomeric cPSTV strands. Synchronous synthesis of single-stranded PSTV was accompanied by a marked increase in dsPSTV RNA, showing its involvement in PSTV replication.

Later on, also dimer and trimer lengths (besides monomers) of PSTV--specific RNAs (+strands) were detected in Northern blots of nucleic acids from PSTV-infected plants (Branch *et al.*, Maramorosch and McKelvey, 1985). Similarly, a set of multimeric (+)strands corresponding to two to five repeats of the ASBV monomer were found in ASBV-infected avocado tissues

(Bruening *et al.*, 1982) and analogous results were achieved for CCCV in coconut plants as well (Haseloff, Mohamed and Symons, 1982).

Taking into account all reliable experimental data, Branch and Robertson (1984) summarized the viroid replication process, in general, as follows:

1) denaturation of viroid RNA (+)strand secondary structure and thus formation of a closed circular template;

2) on this template, synthesis of oligomeric cRNA (−)strands bearing 2 to 5 unit-length transcripts, separated by short spacer sequences;

3) formation of oligomeric linear viroid RNA (+)strands on oligomeric cRNA templates; in vitro, both these steps seem to be directed by DNA-dependent RNA polymerase II, but the RNase engaged in vivo may be different;

4) cleavage of each oligomeric (+)strand (in spacer sequences) into unit-length viroid RNA (+)strands, performed by some endonuclease;

5) immediate circularization of linear monomers; these steps of viroid (+)RNA processing are functions of an RNA ligase.

An alternative model has been suggested by Hutchins *et al.* (1985) on the basis of experiments on ASBV (Bruening *et al.*, 1982), CEV and CCCV, which possibly can apply for most viroid types (species). In this model, the infecting circular (+)RNA of the viroid is copied by a host RNA polymerase to an oligomeric (−)strand which is processed by a non-enzymatic mechanism to a mixture of linear monomers and oligomers. (−)monomers are circularized enzymatically to serve as templates; they are copied, again by a host RNA polymerase, to produce oligomeric (+)strands. Both linear monomers and oligomers are circularized. In this scheme, (−) just as (+)oligomers are considered to arise either by inefficient processing or by head-to-tail secondary ligation of monomers formed, by RNA ligase.

Lin and Semancik (1985) studied the time correlation between host nucleic acid metabolism and citrus exocortis viroid replication in a suspension of host tomato cells. The proportion of CEV RNA concentration to that of host cellular RNA increased towards the stationary phase of cell proliferation. Host RNA synthesis could be inhibited for 80% with actinomycin D which, however, had no effect at all on CEV replication. The proportion of newly synthesized CEV circular and linear forms was surprisingly stable during the host cell proliferation cycle; again, it was shown that both forms are infectious.

Viroid Processing

For viroid processing, a theoretical model considering the recent findings on the primary and secondary structures of viroids, was proposed by Diener (1986). This model is based on the identification of thermodynamically extremely stable base-paired configuration assumed for dimeric or oligomeric viroids or viroid (−)strands. Diener's model presumes that this structure, involving molecular features common to all viroids i.e. the central conserved region and hairpin I are essential for precise cleavage of oligomers and ligation of monomers into circular structures. Because the cleavage-

-ligation process of viroids resembles splicing of nuclear precursor mRNAs to functional mRNAs, the model involves base pairing of complementary sequences close to the future splice site to a thermodynamically stable dimeric (oligomeric) PSTV.

Genetic Engineering in the Study of Viroid Replication

For studies of infectivity, various forms of PSTV molecules were constructed in vitro. PSTV dscDNA was integrated into a plasmid and cloned under the control of a potent promotor (Tabler and Sänger, 1985). Interestingly, in vitro transcribed (—)strands were also infective, but for about 4 ten-orders less than (+)strands.

Another efficient experimental system for the analysis of viroid replication was devised by Gardner, Chonoles and Owens (1986): single-length cDNA of PSTV was integrated into two shuttle vectors of *Agrobacterium tumefaciens* and introduced in them into tomato plant cells. Crown gall tumours containing PSTV cDNA were induced in tomato plants and 10—25 days later, typical symptoms of PSTV infection appeared. In the tissues, syntemic PSTV replication was followed.

Alternatively, viroid replication could be studied in cell protoplasts which had been fused in vitro with liposomes containing encapsulated viroids. Faustmann *et al.* (1985) performed a detailed time-lapse analysis of viroid (+) and (—)strands synthesis in this way and could reveal that the synthesis of oligomeric linear (—)PSTV strands preceded that of the circular (+)PSTV strands, suggesting that the oligomeric (—)strands represented transient linear intermediates of replication. PSTV replication and accumulation was also initiated, if liposomes were used to transfer cloned dsDNA molecules, containing an infectious multimeric PSTV cDNA sequence, to potato protoplasts.

Pathology of Viroid Infection and Its Putative Molecular Basis

Towards the end of the year 1986, viroids were known to cause following plant infectious diseases: tomato bunchy-top (Benson *et al.*, 1965) — agent: TBTV; potato spindle tuber (Diener, 1971*a*) — agent: PSTV; citrus exocortis (Sänger 1972, Semancik and Weathers, 1972) — agent: CEV; chrysanthemum stunt (Diener and Lawson, 1973) — agent: CSV; cucumber pale fruit (Van-Dorst and Peters, 1974) — agent: CPFV; chrysanthemum chlorotic mottle (Romaine and Horst, 1975) — agent: CCMV; coconut cadang-cadang (Randles 1975) — agent: CCCV; hop stunt (Sasaki and Shikata, 1977) — agent: HSV; avocado sun-blotch (Palukaitis *et al.*, 1979) — agent: ASBV; tomato planta macho (Galindo, Smith and Diener, 1982) — agent: TPMV; tomato apical stunt (Walter, 1982) — agent: TASV; burdock stunt (Chen *et al.*, 1983) — agent: BSV (compare Table 2).

Generally, each of these diseases is (certainly or probably) caused by a specific viroid; however, identity appears possible for the tomato bunchy-top and tomato apical stunt viroids, as both tomato infections appear only in Africa, while tomato planta macho — in spite of its high structural homology — is known only in Mexico.

Furthermore, a viroid (CV) has been isolated from apparently healthy *Columnea erytrophae* plants. Having been transferred to potato or tomato plants, it causes pathological symptoms similar to those caused by PSTV; yet the nucleotide sequence of CV is different from that of PSTV.

Similarly, a further viroid (GV) was recently detected (Flores *et al.*, 1985) and isolated (Sano *et al.*, 1986) from grapevines. Its isolates are not pathogenic for grapevine plants, but induce symptoms in cucumber plants indistinguishable from those induced by HSV. The sequence analyses have shown that GV may be a grapevine isolate of HSV. Similarly, CPFV appears to be a cucumber isolate of HSV (see above).

The prevailing symptoms of all viroid-caused diseases do not differ significantly from those of virus diseases: stunting of plants, discoloration and clearing of veins, epinasty (curving of leaves downwards caused by more intensive growth of the upper surface), curling and distortions of leaves, localized chlorotic spots or necrotic spots of rusty-brown shadows, mottling of leaves, necrosis of them etc. The infection leads usually to death of the diseased plants. Many of these symptoms suggest a common primary pathogenic effect.

The research of viroid pathogenic mechanism must respect the fact that some viroids cause diseases in hosts of certain species, but are capable of replication in tissues of other species without inflicting them detectable damage (as already mentioned). Besides, the molecular basis of pathogenicity need not necessarily be the same for all viroids.

Molecular Pathogenicity of Viroids

Since 1979, many putative explanations of the molecular mechanism of viroid action have been formulated. To summarize them briefly, viroids could act as abnormal regulatory molecules causing pathological symptoms by direct interaction with the host genome (Diener, 1971*b* etc.). Alternately, they could compete with the genomic DNA for template binding sites on the RNA polymerase II, thus altering the synthesis of mRNA (Rackwitz, Rohde and Sänger, 1981). Third, they may damage pre-mRNA processing. If viroids did originate, as will be proposed below, by circularization of excized introns of cellular mRNA, their detrimental effects on host cells might be a result of their interference with mRNA maturation processes (Diener, 1981*c*). In detail, some authors considered independently different regions of homology of the sequences of small nuclear RNA U1 (U1 snRNA) to be the key to viroid pathogenesis (Dickson, 1981; Diener, 1981 *c*; Gross *et al.*, 1982). According to these hypotheses, viroids would adopt a secondary conformation permitting them to mimic the putative interaction of U1 snRNA with splice junctions of host cell pre-mRNAs.

For the PSTV group (at least), the pathogenicity is associated with the double-strand conformation of a part of viroid VM region: of the region of mutations between nucleotides 43—44 and 54—56, respectively (Flores, 1984 — see above). Because of the specific conformation of this sequence in different viroids, different ways of their interaction with a specific host cell

protein have been proposed. Generally, it seems plausible that a certain hairpin in viroid precursor concatemer structure is necessary to be distinguished by a cellular endonuclease directing its cleavage into single-length, one-strand viroid RNAs (Meshi *et al.*, 1985).

Schnölzer *et al.* (1985) found two highly conservative sequences in wild PSTV isolates differing by their virulence grades; both are situated in the "left hand" part of PSTV double-strand rods and form their virulence modulating region. Viroids of various virulence differ from each other just by single nucleotide substitutions (insertions, deletions) in the region of mutations of their VM region. Thermodynamical calculations revealed that increase in virulence is accompanied by increasing instability of this region. Regions analogous to virulence-modulating ones in PSTV were proved in all viroids, with exception of ASBV and CCCV (Visvader and Symons, 1986 etc.).

The findings of Rodriguez, García-Martinez and Flores (1978) that in *Gynura aurantiaca* plants infected by CEV a specific substance of auxin character is synthesized, and that the level of endogenous gibberellins is decreased, pointed to a disturbance in a normal metabolism of growth substances.

Epidemiology of Viroid Infections

Different strains of the given viroid type impose cross-immunity to each other on infected plant tissues; also, a weak infection of a plant by a mild viroid makes it non-susceptible to a superinfecting severe strain (Niblett *et al.*, 1978).

All viroid infections are of persistent character: no disease recovery occurs and as long as the plants are alive, viroids can be isolated from them. Generally, the infection spreads horizontally: however, with PSTV at least, also vertical transmission through both pollen and seeds of infected plants has been shown (Fernow, Peterson and Plaisted, 1970; Singh, 1970).

Horizontal transmission occurs by mechanical means, more or less readily. It seems — for PSTV, at least — that the infection is transmitted mainly with farm implements, and the same way may be implicated for other viroids, too (Diener, 1979). No insect vectors have been identified for any viroid.

Origin and Biological Sense of Viroids

Viroids might have been derived from transcripts of eucaryotic insertion sequences or — more sophistically — from transcripts of transposable elements by the loss of their central coding regions and, by at least a partial deletion of their long terminal repeats (Diener, 1983*a*).

The central conserved region of viroids (see above) in its "upper" strand is formed by two nearly complete direct repeats flanked by somewhat incomplete inverted repeats. These patterns are echoed imperfectly in the central conserved region compartment in the "lower" strand. In addition, many further remnants of inverted repeats appear scattered throughout the viroid molecule. Altogether, these striking sequence analogies point to the possible derivation of viroids from transcripts of eucaryotic "jumping sequences".

Another feature, common to PSTV strains at least, is a stretch of 11—18 uninterrupted purine nucleotides centered at position 60 (as mentioned above, too). This sequence reminds of the putative signal site for reverse transcriptase activity in retroviruses. Its presence in viroids, which are not transcribed into (—)DNAs, might reveal a common origin of viroids with retroviruses. The signal site might have become modified for initiating RNA synthesis.

If viroids were really derived from transcripts of transposable elements, one would expect the existence of at least some sequences complementary to those of viroids in the host genomes. However, such sequences have so far not been found.

Thus another hypothesis advanced, independently, by Crick and by Diener as early as in 1979, is accepted as more plausible: that viroids might have originated by the circularization of spliced-out introns of eucaryotic pre--mRNAs. One might speculate that if excised sequences like that permitted extensive intramolecular pairing (as viroids do) and if they got circularized (which viroids are), they might become stabilized and so escape usual degradation. Indeed, circularization of pre-mRNA introns was observed, including some with an approximate size of viroids (Borst and Grivell, 1981). If such introns comprised appropriate recognition sequences, they might be transcribed by a host RNA polymerase and thus escape the control mechanisms of the host cell. A complex of considerable stability is possible between the 5' end of U1 snRNA and nucleotides 257—279 of PSTV (—)strand, covering its hypothetical splice junction between nucleotides 262 and 263 (Diener, 1981). This complementarity may be just a fortuitious coincidence, but the high stability of this complex may support a functional role of this nucleotide sequence in (—)PSTV.

Quite recently, the hypothesis that viroids (and virusoids) originated in escaped introns, has gained an important support by the finding that both share striking structure analogies with transcripts of group I introns (Dinter--Gottlieb, 1986). Group I introns are found in a variety of genes for RNA: in mitochondrial mRNA and rRNA genes, in chloroplast tRNA genes, as in nuclear rRNA genes. The primary structure of these RNAs is characterized by distinct conserved sequences ("boxes") which impose specific secondary and tertiary molecular structure constraints. At least some of these RNAs are capable of self-splicing, i.e. of auto-excision from gene transcripts without the aid of enzymes, releasing small circular RNAs (Netter *et al.*, 1982 etc.).

The close similarity between group I intron transcripts and viroids concerns both their size and structure. Thus, the sequence of 16 nucleotides common to all group I intron RNAs, the "group I consensus", is also contained in the structure of viroids, and so are further boxes governing the two- and three-dimensional structure of these RNAs and — presumably — of viroids as well. Nevertheless, rod-shaped viroid molecules would have to be stabilized by proteins, in order to achieve the three-dimensional disposition of group I intron RNAs. This may, in fact, be the case, as indicated by the finding of PSTV in a ribonucleoprotein complex in vivo (Schumacher, Sänger and Riesner, 1983).

An evolutionary relationship between viroids (virusoids) and RNA introns seems to be revealed with a considerable certainity; but it still remains to be decided whether viroids have evolved from introns or whether both have evolved from a common ancestor RNA molecule.

The Discovery of Virusoids

During the progress of viroid research, a very similar class of low-molecular RNAs taking part in pathogenicity for cultivated plants was found. In virions of velvet tobacco mottle virus, besides its linear viral RN A(signed RNA 1), an additional low-molecular RNA (RNA 2 = VTMoV) was found (Randles *et al.*, 1981; Gould, 1981). This RNA 2 needs the virus genome (RNA 1) as a helper, its own replication and encapsidation being directed by helper, viral RNA-encoded enzymes; thus it may participate in its virus pathogenicity, without having its own one. This is a clear difference from viroids which rely on host cell-encoded enzymes for their replication.

Immediately thereafter, small RNAs of this type were distinguished, encapsidated in virions of two further plant viruses: in *Solanum nodiflorum* mottle virus — here the RNA 2 has been signed SNMV (Gould and Hatta, 1981) and in lucerne transient streak virus (Tien-Po *et al.*, 1981). They all share common features with small satellite circular RNA molecules and are now called virusoids.

In general, virusoids are covalently closed, single-stranded, circular RNA molecules of about 300—400 nucleotides. They bear little sequence homologies with viroids; however, VTMoV and SNMV contain the group I consensus sequence and box sequences which generally occur in viroids and in RNA of group I introns (Haseloff and Symons, 1982).

Animal Viroids and Virusoids

Viroids (and virusoids) are definitely known to occur only in higher plants. However, PSTV RNA has been successfully introduced into *Drosophila* cells in culture by means of liposome fusion (Branch and Robertson, 1984). Therefore, it has been considered reasonable to search for viroids or virusoids in animals and humans suffering from infectious diseases of assumed viral etiology, in which no causative agent has been identified so far. Efforts to find an animal viroid have been untertaken but without any success. Only future work will determine whether viroids indeed are a peculiar feature of plants or whether similar entities exist in other kingdoms of living organisms, as well.

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