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Correlation between structure and pathogenicity of potato spindle tuber viroid (PSTV)

Martina Schnölzer, Bernd Haas, Karla Ramm, Hans Hofmann[1] and Heinz L.Sänger

Abteilung Viroidforschung and [1]Rechenzentrum, Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

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Sequence analysis by primer-extension at the level of their cDNA showed that the RNA genomes of various field isolates of potato spindle tuber viroid (PSTV) of different virulence differ from each other only in a few nucleotides in two distinct regions of the rod-shaped molecule. Despite insertions and deletions the chain length of 359 nucleotides is strictly conserved in all the isolates studied. Thermodynamic calculations revealed that due to the observed sequence differences the region located at the left hand part of the rod-like secondary structure of the PSTV molecule, denoted 'virulence modulating (VM) region', becomes increasingly unstable with the increasing virulence of the corresponding isolate. Based on these data we propose in molecular terms a model for the mechanism of viroid pathogenicity. It implies that the nucleotides of the VM region specify and modulate the binding- and hence the competition-potential of the PSTV RNA molecule for a still unknown host factor(s) and thus determine the virulence of PSTV.

Key words: cDNA sequencing/model for viroid pathogenesis/reverse transcription/thermodynamic analysis/viroid virulence

Introduction

The potato spindle tuber viroid (PSTV) represents the first discovered member of a novel class of plant pathogens (Diener, 1971, 1984). It is a coat protein-free, single-stranded circular RNA chain of 359 nucleotides which exists in its native state as a highly base-paired rod-like molecule. Its structural (Sänger *et al.*, 1976) and thermodynamic (Riesner *et al.*, 1979) properties have been characterized in detail and its primary and secondary structure are known (Gross *et al.*, 1978). Different PSTV field isolates produce disease in tomato plants characterized by mild, intermediate and severe symptoms (Fernow, 1967; Dickson *et al.*, 1979; Gross *et al.*, 1981) and even by the death (Sänger, 1982) of the infected plants. Since RNA is the only component of viroids, differences in the virulence of these isolates can only be based on differences in the sequence of their RNA. The comparison of PSTV RNA fingerprints (Dickson *et al.*, 1979), and of the primary structure of two PSTV isolates of different virulence as obtained by direct RNA sequencing (Gross *et al.*, 1978, 1981) has shown that their RNA sequence differs in a few nucleotides only. However, for a definite assessment of the correlation between viroid structure and viroid pathogenicity, the knowledge of structural details of more than two PSTV isolates is necessary. Therefore we sequenced a series of individual PSTV field isolates of different virulence (Figure 1). These data allowed us to correlate the structural and thermodynamic properties of the RNA genome of these isolates with their virulence for the host plant tomato and to propose a mechanism by which the pathogenicity of PSTV and related viroids could be specified and modulated.

Results

Sequence differences in PSTV isolates

The correlation between primary structure and pathogenicity of viroids cannot be established in a reliable way by sequencing their cloned DNA copies. Because of the well-known microheterogeneity of the sequence of the genomes of RNA viruses (Domingo *et al.*, 1978, 1980; Donis-Keller, 1979; Fields and Winter, 1981; Goelet *et al.*, 1982) which has also been observed in viroids (Visvader and Symons, 1983; Tabler and Sänger, 1984; Tabler *et al.*, 1985), individual molecules may become selected and amplified by cloning which are not necessarily representative for the isolate in question. Therefore, the sequence of the RNA of the PSTV isolates of different virulence was established in a more direct way at the level of their cDNA which was synthesized by primer extension of the PSTV RNA with reverse transcriptase using three PSTV-specific DNA primers (Figure 2). The criteria for choosing these primers and their chemical synthesis are

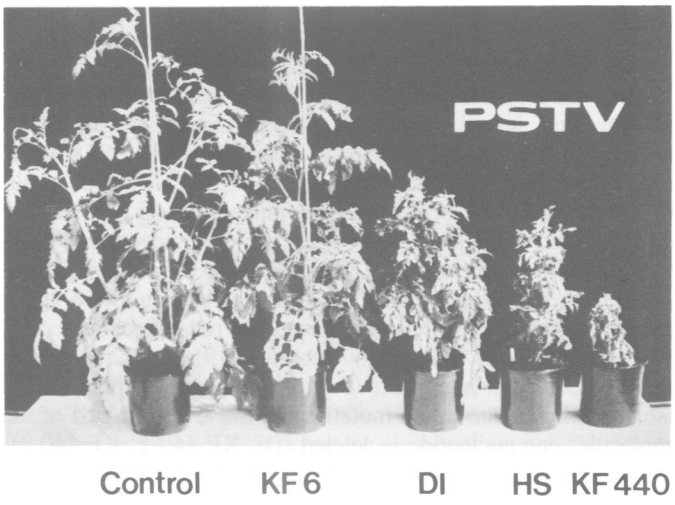
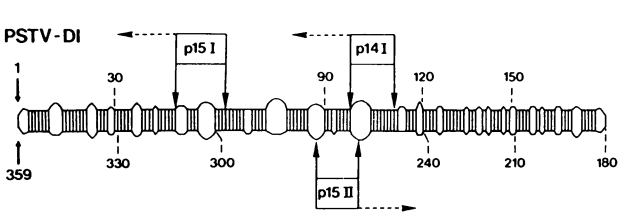


Fig. 1. Tomato plants (cultivar Rutgers) 8 weeks after mechanical inoculation by leaf rubbing with tissue homogenates from tomato plants infected with PSTV field isolates of different virulence. From left to right: uninfected healthy control plant, plants infected with isolates inducing mild (KF 6), intermediate (DI), severe (HS) and lethal (KF 440) symptoms of disease, respectively. The original lethal field isolate KF 440 was found to consist of a population of two RNA molecules of approximately the same concentration. They were designated KF 440-1 and KF 440-2 and differ only in one nucleotide. These two RNA species were separated from each other by molecular cloning; the infectious DNA of isolate KF 440-2 induces the synthesis of the corresponding RNA in tomato plants where a lethal disease was incited (Tabler and Sänger, 1984; Tabler *et al.*, 1985). The virulence of isolate KF 440-1 has not yet been determined because it is not available in an appropriately cloned form. However, our calculations show that this isolate should also induce lethal symptoms (see Figure 5 and text). The origin and abbreviations used for the PSTV isolates are given in Materials and methods.



b

Primer	Sequence (5' → 3')	Binding site at PSTV-DI RNA (nucleotides 5' → 3')
p14 I	d(CGCTCCAGGTTTCC)	97-110
p15 I	d(TTCTTTTTTCTTTTC)	49-63
p15 II	d(GTTTCCACCGGGTAG)	259-273

Fig. 2. Properties of the synthetic DNA primers used for reverse transcription of PSTV isolates. **a,** Hybridization site of the primers at the (+)RNA of the PSTV strain DI. The direction of reverse transcription is shown by broken arrows. **b,** Sequence of the primers and the region to which they are complementary.

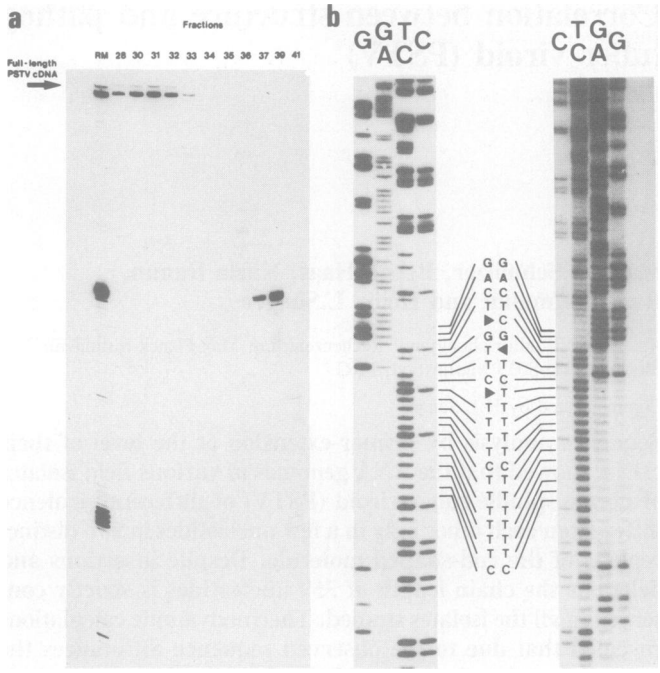


Fig. 3. Determination of the primary structure of PSTV isolates at the cDNA level. **a,** Analysis on an 8% polyacrylamide (PA) gel of the fractions obtained by gel permeation chromatography of the primer p15II-initiated HS cDNA. **b,** Sequence analysis of the primer p14I-initiated cDNA of PSTV isolates KF 440 (left hand side) and HS (right hand side) on an 8% PA sequencing gel showing the upper strand of the region where the sequence of these two isolates differs in three nucleotides as marked by arrowheads. The details of reverse transcription of PSTV RNA and of sequencing of the corresponding PSTV cDNA are given in Materials and methods.

described in Materials and methods. The PSTV cDNAs obtained by reverse transcription were then sequenced with the Maxam-Gilbert technique (Figure 3). This approach was possible because in all the cases presented here the majority of the PSTV molecules of a given isolate was represented by one RNA species. Under the assumption that the population of these predominating RNA molecules is responsible for the phenotype of the disease observed, the sequence obtained represents the 'master sequence' (Eigen, 1971; Eigen and Schuster, 1977; Biebricher, 1983) of the corresponding PSTV isolate (Tabler *et al.*, 1985).

The sequence analysis of seven PSTV isolates revealed that substitutions, insertions and deletions of nucleotides are found at three distinct sites of the primary structure of the PSTV RNA molecule (Figure 4a). As compared with the PSTV type strain (PSTV DI) they are located between nucleotides 45 – 50, 119 – 122 and 308 – 318, respectively. Regarding the secondary structure model, nucleotides 45 – 50 and 308 – 318 are partly complementary and positioned opposite to each other in the upper and lower strand in the left hand part of the rod-shaped molecule, whereas the region between nucleotides 119 and 122 is located in its upper right hand part (Figure 4a,b). In all those PSTV isolates where, due to the mutations in the left hand part of the molecule, one nucleotide is deleted (DI, KF 440-1, KF 440-2), this deletion is always compensated for by the substitution of one uridine by two adenosines at position 120/121 in the right hand part (Figure 4a,b) so that the total number of the 359 nucleotides of the PSTV RNA genome is strictly maintained. It has been reported that the RNA of a severe PSTV isolate from Scotland (Harris and Browning, 1980) as sequenced after molecular cloning, consists of 358 nucleotides (van Wezenbeek *et al.*, 1982) but we found with our sequencing strategy that this isolate (PSTV-DS according to our nomenclature) has also a chain length of 359 nucleotides.

The occurrence of the mutations in only two distinct domains of its rod-shaped molecule seems to be a special feature of PSTV. In the case of citrus exocortis viroid (CEV) where the sequence of five isolates has been established (Visvader and Symons, 1983; Visvader *et al.*, 1982; Gross *et al.*, 1982) only in the isolate DE 26 did the mutations predominate in two regions (Visvader and Symons, 1983), but additional single mutations are scattered over the entire RNA of all the isolates. However, the chain length with 371 nucleotides is also strictly conserved in all the CEV isolates described so far. The reason for this conservation is unknown but the chain length of the non-translated RNA molecule *per se* is certainly not an essential feature for the replicability of viroids in one and the same host plant. Two isolates of chrysanthemum stunt viroid (CSV) were found to consist of 354 and 356 nucleotides, respectively (Gross *et al.*, 1982; Haseloff and Symons, 1981) and four individual viroid 'species', the tomato apical stunt viroid, TASV, (Kiefer *et al.*, 1983) and the tomato plant macho viroid, TPMV (Kiefer *et al.*, 1983), both with a chain length of 360 nucleotides and hop stunt viroid (HSV) with 297 nucleotides (Ohno *et al.*, 1983) and cucumber pale fruit viroid (CPFV) with 303 nucleotides (Sano *et al.*, 1984), are replicated in the same host plants as PSTV with its 359 nucleotides. In the case of coconut cadang cadang viroid (CCCV) even four RNA species 246, 287, 492 and 574 nucleotides long and generated from the 246 nucleotide monomer by certain sequence duplications are replicated in coconut palms (Haseloff *et al.*, 1982).

Nucleotide changes and secondary structure

The influence of the observed nucleotide changes on thermodynamic parameters of the secondary structure of the corresponding PSTV isolates was evaluated by thermodynamic calculations using two computer programs described under Materials and methods. This approach allows the determination of the influence of small sequence differences on the secondary structure of the PSTV RNA molecule. The observed mutations induce only local

structural changes at the site where they occur without influencing the overall secondary structure of PSTV. Only the changes in the left hand part of the molecules (Figure 4b) seem to be functionally important for the virulence of PSTV because due to them the thermodynamic stability of this region becomes different in the isolates of different virulence.

Previous thermodynamic studies on the PSTV type strain (PSTV-DI) have shown that its rod-like shape is maintained up to temperatures close to its rather sharp melting temperature (Riesner *et al.*, 1983b; Steger *et al.*, 1984). However, there are three so-called 'pre-melting (PM) regions' in the molecule, which are already transformed below this melting temperature into three inner loops called the 'pre-melting (PM) loops' 1, 2 and 3 (Figure 5a). PM loop 1 is formed between nucleotides guanosine 49-adenosine 60 in the upper and between nucleotides uridine 300-uridine 312 in the lower strand and comprises a part of the region where the mutations predominate. The upper strand of PM loop 1 is characterized by a polypurine sequence whereas the lower strand contains the palindromic heptanucleotide sequence 3′-UCUAUCU-5′ (Figure 4c). Under the presumption that the observed mutations modulate the virulence of the PSTV isolates by changing the thermodynamic behaviour of the region of PM loop 1 we denote the region of PM loop 1 together with the region of mutation the 'virulence-modulating (VM) region' of PSTV (Figures 4c and 5a). The free energy to form PM loop 1 in the structures shown in Figure 4c was calculated for 25°C and 1 M NaCl. It was found to be 4.51, 2.53, 0.75 and 0.43 kcal/mol for the mild, intermediate, severe and lethal isolates, respectively. PM loop 2 which is formed between nucleotides guanosine 73-cytosine 93 and guanosine 266-cytosine 285 in the upper and lower strand, respectively, comprises parts of the conserved central region of the PSTV molecule and is therefore found in most viroid species. Its strict conservation indicates that this region is of functional importance and might be essential for viroid replication. PM loop 3 of PSTV is located between guanosine 134-guanosine 143 in the upper strand and cytosine 218- cytosine 228 in the lower strand and conserved in all PSTV isolates. Such a pre-melting region exists at the same position in most other viroids although the corresponding sequence is not conserved. In the case of CEV this pre-melting region is, in fact, characterized by the accumulation of nucleotide changes when the CEV isolate DE 26 is compared with the four other known CEV isolates and with PSTV. Regarding the function of all these pre-melting regions one could postulate that they provide the basis for specific structural rearrangements of the viroid RNA genome and contribute to its functional flexibility.

The compensating nucleotide change at the right hand side of the PSTV RNA where one uridine is replaced by two adenosines (Figure 4b) slightly increases the stability of this region *via* extending the helix by 1 bp and by concurrently decreasing the adjacent internal loop at the left hand side of this short helix by one nucleotide.

Concentration, melting behaviour and virulence of PSTV

Differences in PSTV virulence could be primarily based on the amount of PSTV RNA accumulating in the host plant tissue after infection. The observed sequence differences could, for example, influence viroid synthesis and modulate disease in this way. Therefore, we analyzed at different times after inoculation the PSTV RNA content in comparable newly developing leaves of tomato plants infected with the various PSTV isolates. No significant differences in the PSTV RNA content were found between the isolates of different virulence when the corresponding RNA samples were separated in bi-directional runs on polyacrylamide gels (Schumacher *et al.*, 1983a) and compared after visualization by silver staining (data not shown). Consequently, another mechanism of PSTV disease modulation must operate.

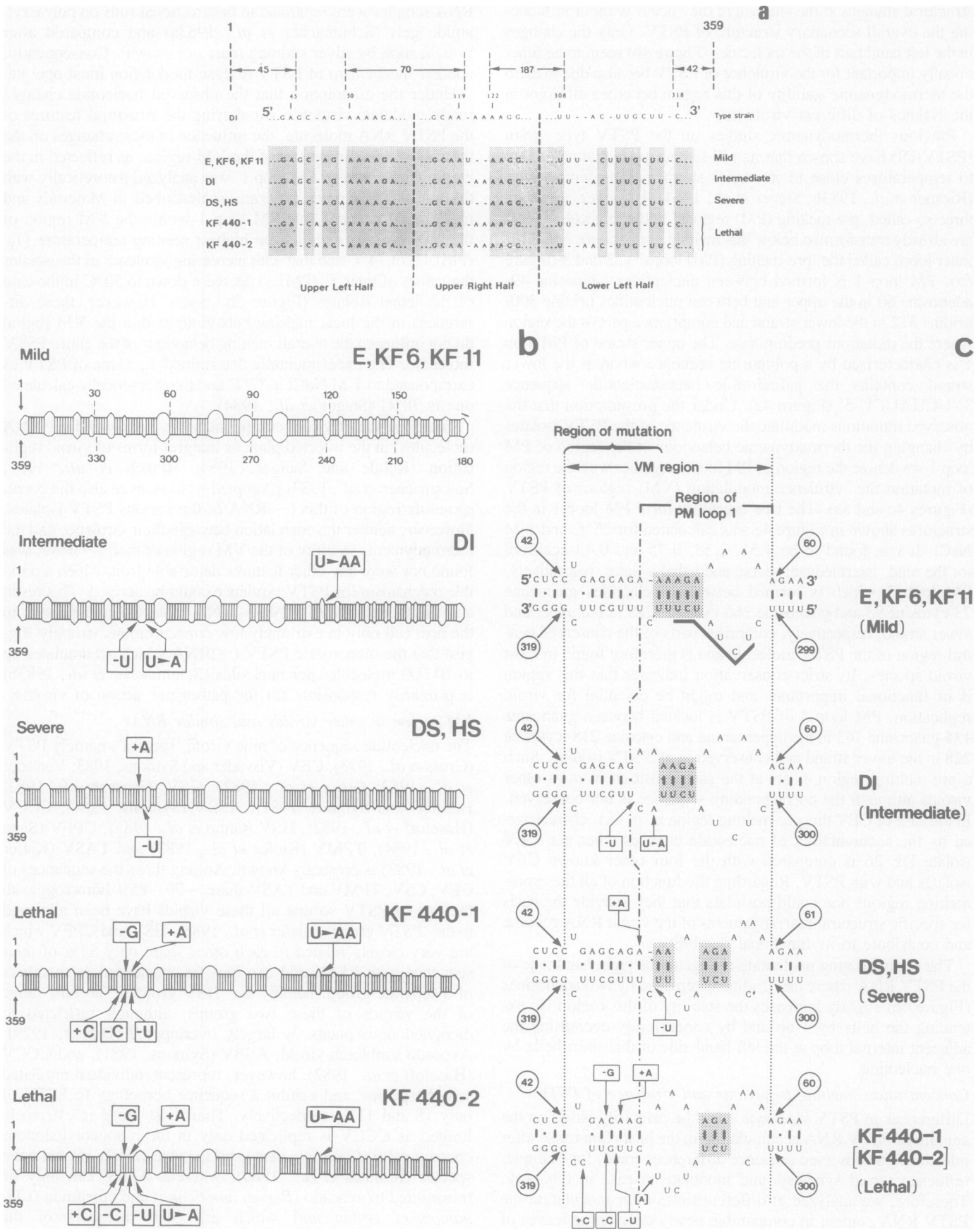
Under the assumption that the observed nucleotide changes might modulate virulence *via* altering the structural features of the PSTV RNA molecule, the influence of these changes on the thermodynamic properties of the VM region, as reflected in the melting behaviour of PM loop 1, was analyzed theoretically with the aid of a computer program as described in Materials and methods. It is found that PM loop 1 within the VM region of the mild isolates exhibits the highest melting temperature [T_m (PML1)] of 74°C and that with increasing virulence of the isolates the values of their T_m(PML1) decrease down to 50°C in the case of the lethal isolates (Figure 5b, inset). However, these differences in the local melting behaviour within the VM region do not influence the overall melting behaviour of the entire PSTV molecule. The experimentally determined T_m value of PSTV as extrapolated to 1 M NaCl is 77°C and the theoretically calculated one is 78°C (Steger *et al.*, 1984).

The presence of viroid complementary, i.e., viroid (−)RNA molecules, in the infected plant as integral forms of viroid replication (Rohde and Sänger, 1981; Branch *et al.*, 1981; Spiesmacher *et al.*, 1983) prompted us to analyze also the corresponding region of this (−)RNA of the various PSTV isolates. However, neither the correlation between their virulence and the thermodynamic stability of the VM region of their (−)RNA was found nor were any other features detectable from which a possible mechanism for PSTV virulence could be derived. This result and the fact that the PSTV (−)RNA molecules are present in the host cell only in extremely low concentrations strongly suggest that the monomeric PSTV (+)RNA which accumulates up to 10 000 molecules per nucleolus (Schumacher *et al.*, 1983b) is primarily responsible for the pathogenic action of viroids.

VM region in other viroids and similar RNAs

The nucleotide sequence of nine viroid 'species', namely PSTV (Gross *et al.*, 1978), CEV (Visvader and Symons, 1983; Visvader *et al.*, 1982; Gross *et al.*, 1982), CSV (Gross *et al.*, 1982; Haseloff and Symons, 1981), ASBV (Symons, 1981), CCCV (Haseloff *et al.*, 1982), HSV (Ohno *et al.*, 1983), CPFV (Sano *et al.*, 1984), TPMV (Kiefer *et al.*, 1983) and TASV (Kiefer *et al.*, 1983) is presently known. Among these the sequences of CEV, CSV, TPMV and TASV share ~70−85% homology with the one of PSTV so that all these viroids have been allocated to the 'PSTV group' (Kiefer *et al.*, 1983). HSV and CPFV which are very closely related to each other share only 55% of their sequence with PSTV and can thus be considered as members of a separate group, namely the 'HSV group'. The host range of the viroids of these two groups, although restricted to dicotyledonous plants, is largely overlapping (Diener, 1979). Avocado sunblotch viroid, ASBV (Symons, 1981), and CCCV (Haseloff *et al.*, 1982), however, represent individual unrelated viroid 'species' and exhibit a sequence homology to PSTV of only 18 and 11%, respectively. Their host range is extremely limited as CCCV is replicated only in the monocotyledonous coconut palm (*Cocos nucifera*) and in some closely related palm species (Randles *et al.*, 1980), whereas ASBV can only be transmitted to avocado (*Persea americana*) and cinnamon (*Cinnamomum zeylanicum*) which are both members of the dicotyledonous family Lauraceae (Da Graca and Van Vuuren, 1980).

With the exception of ASBV and CCCV a domain similar to


a
1
359
45
70
187
42
5'
3'
DI
Type strain
E, KF6, KF11
DI
DS, HS
KF 440-1
KF 440-2
Mild
Intermediate
Severe
Lethal
Upper Left Half
Upper Right Half
Lower Left Half
b
c
Mild
E, KF6, KF11
Intermediate
DI
Severe
DS, HS
Lethal
KF 440-1
Lethal
KF 440-2
U►AA
-U
+A
-G
+C
-C
Region of mutation
VM region
Region of PM loop 1
E, KF6, KF11 (Mild)
DI (Intermediate)
DS, HS (Severe)
KF 440-1 [KF 440-2] (Lethal)

the VM region of PSTV can be detected in all viroids, at the same topological position of their rod-shaped secondary structure as shown for PSTV in Figure 4b. It exhibits a high degree of sequence homology in particular to the region of PM loop 1 of PSTV with its purine-rich sequence in the upper (Figure 6a) and the characteristic palindromic 3′-UCUAUCU-5′ sequence in the lower strand (Figure 6b), respectively. In ASBV this homology to the VM region is only partial and restricted to the lower strand of its RNA structure whereas in CCCV no homology is detectable at all. This is not too surprising because CCCV and ASBV also cause a completely different type of disease in their hosts which is characterized by yellowing type symptoms in contrast to the growth retardation and leaf malformation incited by the other viroids. Moreover, CCCV and ASBV are evidently not associated with the cell nucleus like PSTV (Spiesmacher *et al.*, 1983, 1985; Schumacher *et al.*, 1983b) and the others, but predominantly with the cytoplasm and its reticulum (CCCV) (Randles *et al.*, 1976) or with the chloroplasts (ASBV) (Mohamed and Thomas, 1980). From all these differences one could expect that the mechanism of pathogenesis of these two viroids [and probably also their site and mode of replication (Spiesmacher *et al.*, 1985)] might differ from the one we have developed here for PSTV and its relatives. Since no comparative studies are available, as yet, on the correlation between the thermodynamic parameters of the virulence of different isolates of the other members of the PSTV and HSV group, it is still unknown whether or not their virulence is also specified and modulated by this domain as we propose for PSTV.

No domain similar to the VM region of PSTV can be detected in the so-called virusoids which are satellite RNAs of certain plant viruses and viroid-like in size and secondary structure although they show only ~1% sequence homology to the viroids (Gould, 1981; Gould and Hatta, 1981; Tien *et al.*, 1981; Jones *et al.*, 1983). However, a stretch of six nucleotides (3′-CUAUCU-5′) of the palindromic heptanucleotide sequence present in the VM region of the viroids is also found near the 3′ end of the cucumber mosaic virus (CMV)-associated small 334–336-nucleotide satellite-like CARNA 5 RNAs (Richards *et al.*, 1978; Collmer *et al.*, 1983a, 1983b; Palukaitis *et al.*, 1983; Palukaitis and Zaitlin, 1984). These CARNA 5 satellite RNAs are dependent upon CMV for their replication and modulate the disease expression of their helper virus (Kaper and Waterworth, 1977) in a trilateral interaction of extreme specificity (Kaper, 1982; Kaper and Tousignant, 1984).

Discussion

Several lines of evidence suggest that viroids are not translated (Davies *et al.*, 1974; Hall *et al.*, 1974; Semancik *et al.*, 1977) and the accumulation of certain proteins in tomato plants infected with PSTV (Zaitlin and Hariharasubramanian, 1972; Camacho and Sänger, 1982a, 1982b, 1983; Galindo *et al.*, 1984) and in *Gynura aurantiaca* infected with CEV (Conejero and Semancik, 1977; Flores *et al.*, 1978; Conejero *et al.*, 1979) is not a viroid-specific response but a general pathophysiological reaction of the host to infection and also induced by conventional viruses and fungi (Camacho and Sänger, 1982a, 1982b, 1983). Therefore, there is general agreement that viroid pathogenicity can only be exerted by the viroid RNA genome itself.

It has been postulated that viroid RNA might act as an aberrant kind of autoinducing 'regulatory RNA' (Diener, 1971, 1977, 1979; Semancik and Weathers, 1972; Robertson and Dickson, 1974; Reanny, 1975; Dickson and Robertson, 1976; Dickson, 1979; Zimmern, 1982) and cause disease by disregulating the expression of host genes. From the total dependence of viroid replication on host enzymes it has also been surmised that viroid RNA may incite disease by usurping the corresponding DNA-dependent RNA polymerases for its 'selfish' replication (Rackwitz *et al.*, 1981). On the basis of certain sequence homologies it has furthermore been argued that the viroid RNA and/or its complement interferes with the components and mechanisms involved in pre-mRNA splicing (Gross *et al.*, 1982; Diener, 1981; Dickson, 1979) thereby perturbing normal mRNA processing. From the disease symptoms induced by viroid infections such as growth retardation and stunting of plants combined with curling and malformation of their leaves it has been concluded that viroids induce disturbances in the metabolism of plant growth hormones (Rodriguez *et al.*, 1978; Semancik, 1979; Duran-Villa

Fig. 4. Primary and secondary structure of PSTV isolates of different virulence. All the secondary structures were obtained by a computer program as described in Materials and methods. **a,** The nucleotide differences between the various PSTV isolates as shown on the aligned primary structure of their RNA. The sequences were established at the level of the primer-extended cDNA as described in Figure 3. Only those regions in which the sequence of the isolates differs are shown. The shadowed areas represent the regions of nucleotide homology between which the observed changes are located in the different isolates. The primary structures are ordered according to the increasing virulence of the isolates. The nucleotide changes are limited to three distinct sites which are shown in relation to the PSTV type strain (PSTV-DI) and its nucleotide numbering. −, gap introduced into the sequence to obtain a maximal degree of homology. The abbreviations used for the PSTV isolates and their origins are described in Materials and methods. **b,** Location of the nucleotide changes at the secondary structure model of the PSTV isolates causing mild, intermediate, severe and lethal disease symptoms in Rutgers tomato as shown in Figure 1. The changes are indicated in boxes and refer to the mild isolate. **c,** Differences in the secondary structure of the VM region of PSTV isolates of different virulence as generated by the nucleotide changes observed in the region of mutation. The changes indicated in boxes refer to the mild isolates. The palindromic heptanucleotide sequence 3′-UCUAUCU-5′ present in the lower strand of the region of PM loop 1 of all isolates is underlined by a black bar in the mild isolates only. The shadowed base pairs between the nucleotides of the upper and lower strand are the ones which dissipate when pre-melting (PM) loop 1 is formed. From the decreasing number of these base pairs in this region it is evident that the energy required for the melting of this region decreases with increasing virulence of the PSTV isolates. In the case of KF 440-1 a structure in the region of PM loop 1 exists which contains the helix consisting of the 4 bp $\overset{AAGA}{UUCU}$ instead of three. However, this structure is thermodynamically unfavoured because of the destabilization energy contributed by the purines (Riesner *et al.*, 1979) in the adjacent inner loop at the left hand side. The secondary structure in which this 4-bp helix is reduced to a helix consisting of the 3 bp $\overset{AGA}{UCU}$ has the same free energy value as the structure shown in the figure. Therefore, it represents an alternative structural isomer of lowest free energy. It should be noted in this context that all PSTV isolates can adopt a secondary structure in the region of the PM loop 1 which is characterized by an inner loop on its left hand side which comprises nine or ten nucleotides and on its right hand side by an inner loop of five nucleotides. This structural isomer is virtually identical to the region of the PM loop 1 of the lethal isolates KF 440-1 and KF 440-2 shown in the figure. The calculated free energy value to form this structural isomer from the one of lowest free energy can be correlated with the virulence of these isolates. This value is 4.08 kcal/mol for the mild, 2.26 kcal/mol for the intermediate, 0.32 kcal/mol for the severe and 0 kcal/mol for the lethal PSTV isolates, respectively. However, the thermodynamic analysis of the putative VM region of the other viroids revealed that these cannot form identical structural isomers although some of them, like TASV and TPMV, incite a lethal disease in tomato. Therefore, we favour a model which invokes PM loop 1 for the modulation of PSTV virulence.

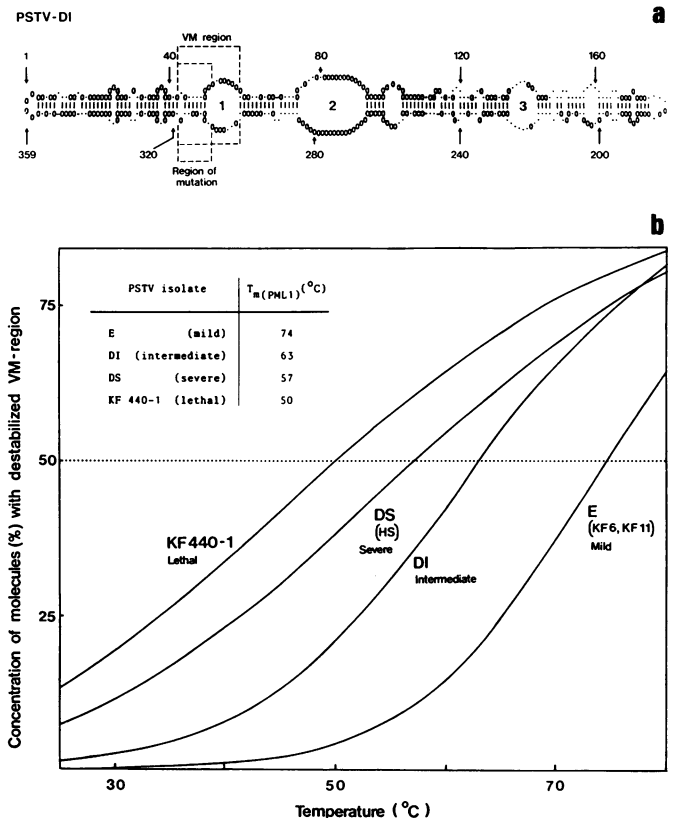


Fig. 5. Thermodynamic analysis of the melting behaviour of PSTV RNA. **a,** Calculated secondary structure of PSTV-DI RNA according to the model of cooperative single helices (Steger *et al.*, 1984) at 70°C which is 8°C below the main calculated melting point. The nucleotides represented by open circles are identical in the three viroid 'species' PSTV, CEV and CSV, the varying ones are represented by points. In contrast to the native structure the pre-melting structure of PSTV exhibits three larger inner PM loops (indicated by numbers within the structure) of which PM loop 1 and 2 are characterized by nucleotides conserved also in CEV and CSV (Steger *et al.*, 1984). The region of mutation in PSTV (see also Figure 4) comprises a part of the region of PM loop 1. As explained in the text, both regions together are defined as the VM region of PSTV. **b,** Computed melting curves characteristic for the VM region of various PSTV isolates and the melting temperature T_m(PML1) (see inset) at which 50% of the population of viroid molecules exhibit at least the nucleotides of PM loop 1 (see Figure 4c) in an unpaired form. This value reflects the structural stability of the VM region of the different PSTV isolates. It is found that the T_m(PML1) values and hence the stability of the VM region of the isolates decreases with their increasing virulence. The curve for the lethal KF 440-2 is not shown because it is practically identical to the one shown for KF 440-1. The abbreviations used to denote the PSTV isolates are explained in Materials and methods.

and Semancik, 1982). As has been discussed in detail (Diener, 1983; Flores, 1984) none of these models including the most recent one of Flores (1984) can readily explain in molecular terms how a viroid specifies and modulates disease and why it is pathogenic in some hosts yet harmless in others.

Model for the pathogenic action of PSTV and its modulation

We have found that the pathogenicity of PSTV can be correlated with nucleotide changes in a distinct region of the PSTV RNA genome. This has led us to the assumption that the virulence of the PSTV isolates is specified and modulated by the nucleotides in this so-called VM region. We furthermore found a correlation between the severity of these isolates and the ease with which the region of PM loop 1 which is a part of the VM region can be melted *in vitro*. Therefore we wondered whether this property is related to the induction of disease by the various PSTV isolates *in vivo*. If so, the most plausible assumption would be that the nucleotides of the VM region of PSTV interact in some way with the still unknown host factor(s) and thus incite disease. Since the thermodynamic stability of the region of PM loop 1 decreases with increasing virulence one can conclude that concurrently the binding of the host factor(s) becomes more probable which would aggravate the resulting disease. The observed correlation would also indicate that the host factor(s) is apparently specific for a single-stranded part of the VM region. Moreover, the binding site for the host factor(s) should comprise in all PSTV isolates the same nucleotides in the conserved nucleotide sequence of PM loop 1. Under these premises the observed nucleotide changes in the region of mutation would specify and modulate PSTV virulence by altering the accessibility of the target sequence for the host factor(s) at the PSTV RNA molecule. It follows that the nucleotides of the upper or lower strand of PM loop 1 are most probably the only site where the binding of the host factor(s) can take place. However, our present data do not allow us to decide to what extent the nucleotides of the double-stranded regions adjacent to the left and right hand side of PM loop 1 are also involved in this interaction. Moreover, the nature of the putative host factor(s) is also unknown as yet.

Assuming that the VM region is recognized by a protein, this could bind to the nucleotides of the lower strand of PM loop 1 in a stable tertiary structure which would be favoured by the stacking of the polypurine stretch in the corresponding upper strand. In cases where a nucleic acid would base pair with the nucleotides of PM loop 1 the resulting binding energy would not be sufficient to 'titrate out' this nucleic acid factor. However, the extensive homology present at both sides of the region of PM loop 1 in all viroids except CCCV and ASBV (Figure 6) would suggest that such a nucleic acid might be complementary to a much longer sequence in this region and hence exhibit a high affinity for the viroid molecule. Such a complex could of course become further stabilized by an additional host protein.

The interaction between the PSTV RNA and the putative host factor(s) could be influenced not only by parameters which determine the state of equilibrium but also by the activation energies which define the kinetics of how the equilibrium is reached. Therefore, the VM region with or even without the bound host factor(s) could exert its virulence-modulating function in that it initiates a structural transition of the viroid RNA molecule. Viroid sequences might then become exposed in structures such as bulge loops, distorted helices or hairpins all of which have been shown to be responsible for the recognition and binding of proteins by RNA molecules (Gralla *et al.*, 1974; Krug *et al.*, 1982; Carey *et al.*, 1983; Garret *et al.*, 1981). Although the actual structure of viroids *in vivo* is unknown, the unique thermal melting behaviour of the rod-shaped PSTV RNA molecule with its various transient metastable and intermediate structures (Riesner *et al.*, 1983a, 1983b; Steger *et al.*, 1984; Zuker and Stiegler, 1981) suggests that such structural rearrangements are not only potentially possible but they probably reflect an essential feature of functional importance for this unique class of pathogenic small circular RNAs.

The predominant occurrence of mutations in one part of the VM region of PSTV suggests that the corresponding nucleotides are not essential for the 'survival' of viroids. In fact, our model implies that pathogenicity and replicability are controlled by different domains of their RNA genome. From the strict conservation of the central region in all viroids (Gross and Riesner, 1980;

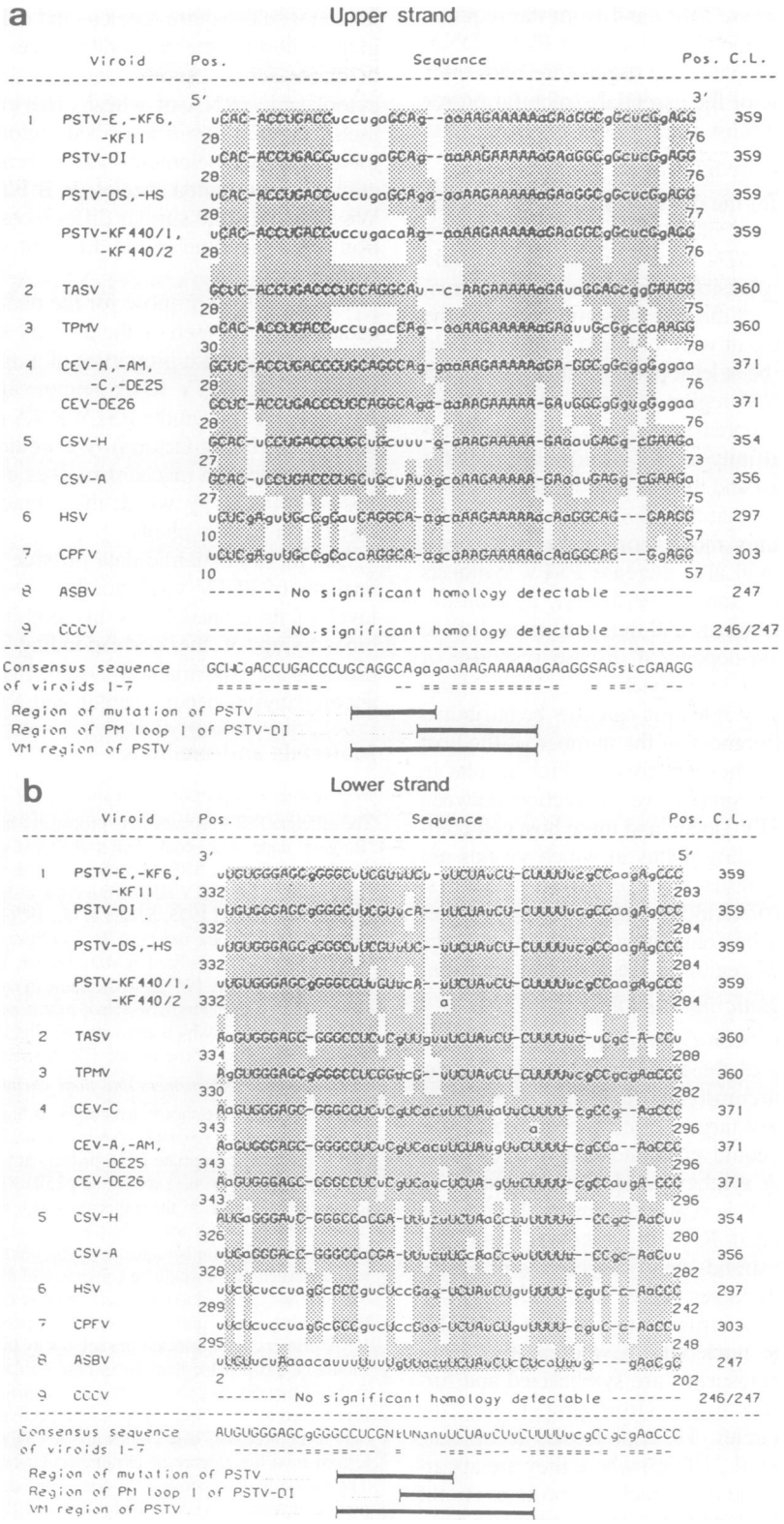


Fig. 6. Comparison of the nucleotide sequence of the VM region of PSTV isolates with the corresponding sequences in all other viroids presently known in the upper strand **(a)** and in the lower strand **(b)** of their secondary structure model. All shaded nucleotides are identical to the nucleotides of the consensus sequence. The region of mutation, PM loop 1 and the VM region of PSTV are shown at the bottom of each comparison by black bars. Pos., position of the 5′ and 3′ end, respectively, of the depicted region within the total sequence; C.L., chain length given as the number of nucleotides of the corresponding single-stranded circular viroid RNA genome; −, gap introduced into the sequence to obtain a maximal degree of homology, A, C, G and U denote the four common ribonucleotides whereas the letters K, S, W and N in the consensus sequence denote positions where G or U, C or G, A or U, and any of the four nucleotides, respectively, can occur. Details on how the consensus sequence was established, and on the meaning of capital and lower case letters and the underlining of certain nucleotides are given in Materials and methods. The abbreviations for the different isolates of PSTV are also given in Materials and methods whereas the ones for the CEV isolates are explained by Visvader *et al.* (1983). CSV-H is an isolate from the UK (Gross *et al.*, 1982) and CSV-A is an Australian isolate sequenced by Haseloff and Symons (1981).

Sänger, 1982; Riesner and Gross, 1985) and from the requirement of its presence for the infectivity of cloned PSTV DNA (Tabler and Sänger, 1984, and unpublished results) one must infer that this central domain is one of the essential structural prerequisites for the replication and subsequent processing of viroids.

Implications of the pathogenicity model

Our model is able to account for the modulation of PSTV-incited disease symptoms and for several other phenomena specific for the development of disease in PSTV-infected plants which could not be interpreted so far in a plausible way in molecular terms. (i) The increase in the severity of the disease symptoms with the increasing ambient temperature at which tomato plants are held after PSTV infection can now be at least partly related to its direct effect on the stability of the VM region of the PSTV molecule. It becomes destabilized with increasing temperature which increases in total the binding affinity of the viroid molecules for the putative factor(s) of the host and thus its pathogenic response. It should be stressed, however, that the effect of temperature on symptom expression is certainly much more complex because any increase in temperature will also increase PSTV synthesis and accumulation (Sänger and Ramm, 1975) and, in addition, directly affect host plant metabolism and thus influence disease development. (ii) The cultivar-dependent disease response of potato (Diener, 1979) and tomato (Mühlbach *et al.*, 1977; Mühlbach and Sänger, 1977) to PSTV infection can now be attributed to genetically determined differences in the number of the host factor(s) or its substrate(s) in these cultivars which results in quantitative differences in the competitive interaction between the RNA molecules of the PSTV isolate and these host cell components. (iii) In all non-responding plants in which viroids are well replicated without causing disease, the putative host factor(s) responsible for the induction of viroid pathogenesis could either be present in large excess, or be completely absent, or lack the affinity for binding to the VM region of the viroid in question.

The host plant in the pathogenic interaction

Our pathogenicity model has been derived from the structural and thermodynamic analysis of the RNA genome of different PSTV field isolates. Only circumstantial evidence is available so far for the nature of the host target which is required for the pathogenic interaction. One could postulate that it takes place at the site of PSTV (+)RNA synthesis and accumulation. We could show recently that PSTV is replicated inside the nucleus and that the two DNA-dependent RNA polymerases I and II of the host are involved in the strand-specific synthesis of PSTV (+) and (−)RNA, respectively (Spiesmacher *et al.*, 1983, 1985). The location of these RNA polymerases suggests that the PSTV (−)RNA is generated in the nucleoplasm whereas the linear oligomeric PSTV (+)RNA transcripts are synthesized and further processed in the nucleolus to the viroid proper, i.e., the monomeric PSTV (+)RNA circles. These molecules accumulate in the nucleoli (Schumacher *et al.*, 1983b) where they are apparently complexed with histones and other nucleolar proteins (Wolff *et al.*, 1985) which constitute, together with the genomic ribosomal DNA, the nucleosomal structure. Therefore, PSTV and presumably also its relatives seem to exert their pathogenic action inside the nucleolus where they could interfere with the transcription of the cytoplasmic rRNAs. It is tempting to speculate that promoter and enhancer elements at the genomic rDNA and/or the corresponding regulatory proteins are the targets with which the viroid RNA can interact. The different response of the various species and cultivars of tomato (Sänger and Ramm, 1975; Mühlbach *et al.*, 1977) and potato (Diener, 1979) to PSTV infection would require species- and cultivar-dependent differences in these host targets. Such differences have, in fact, been detected in plants at the genomic level in the multigene family of the cytoplasmic rRNA of wheat. The total number of these genes including their non-transcribed regions with their repetitive promoter/enhancer elements varies remarkably in various wheat cultivars and related species (R.B.Flavell, unpublished results). We anticipate that similar differences also exist in the organization of the genome of the different viroid host plants and their cultivars.

The model we propose for the pathogenicity of PSTV in molecular terms is based on the primary and secondary structure and the thermodynamic properties of a distinct virulence-modulating region of the PSTV RNA genome and invokes the competitive interaction between the PSTV RNA molecule and a specific but still unknown host factor(s). We would like to point out, however, that the proposed mechanism does not pretend to exclude other possible modes by which these unique pathogens might incite disease in higher plants.

Our thermodynamic data provide the rationale for the direct analysis of PSTV virulence by site-specific mutagenesis at the level of its cloned DNA in combination with the subsequent bioassaying of the corresponding *in vitro*-synthesized RNA utilising an experimental system like the one described in our accompanying paper (Tabler and Sänger, 1985).

Materials and methods

Propagation and partial purification of the PSTV isolates

The different PSTV isolates were propagated and bioassayed in the tomato cultivar 'Rutgers' under semi-controlled greenhouse conditions as previously described (Tabler and Sänger, 1984). Phenol extraction of total nucleic acids and partial purification of the PSTV RNA from it followed essentially the described protocols (Sänger and Ramm, 1975; Sänger *et al.*, 1976). The abbreviations for the isolates are either related to the names of the investigators who provided the original culture [DI: Dr T.O.Diener, Belstville, MD, (Diener, 1971); KH: Dr K.H.Fernow, Ithaca, NY, USA (Fernow, 1967)], to the name in combination with the virulence [HS: Dr P.S.Harris, Edinburgh, Scotland, severe isolate (Harris and Browning, 1980)], to the country from which they came (E: England) or the country in combination with the virulence of the isolate (DS: Dutch, severe isolate).

PSTV-specific DNA primers and their chemical synthesis

Previous studies have shown that despite its highly base-paired secondary structure, PSTV RNA can be reversely transcribed into cDNA molecules in the size range of full length if appropriate DNA primers are used (Rohde *et al.*, 1981a, 1981b). Two out of three primers (p14I and p15II) were chosen so that they would bind to the central region of the molecule which is strictly conserved in three viroid 'species' PSTV, CEV and CSV (Gross *et al.*, 1982). Assuming that the conservation of this domain is related to a still unknown but important viroid function we anticipated that it would be conserved all the more in the different field isolates of PSTV we intended to sequence. Because of the high degree of intramolecular base pairing in this part of the central region of the PSTV RNA molecule, the DNA primers were selected in such a way that the resulting DNA-RNA hybrid should be more stable than the intramolecular base pairing of viroid RNA itself at the primer binding site. In the case of primers p14I and p15II, strong binding is favoured by nine G-C base pairs, respectively. The additional primer p15I can only form three G-C base pairs with the PSTV-DI RNA but this region is characterized by a low degree of intramolecular base pairing of the viroid molecule. The deoxyoligonucleotide p15I was synthesized by the modified phosphotriester method in solution as described previously (Rohde *et al.*, 1981a). The synthesis of the primers p14I and p15II was carried out according to the phosphite triester method on a polymer support (Matteucci and Caruthers, 1981; Beaucage and Caruthers, 1981). The primers were purified by two h.p.l.c. runs before and after deblocking of their 5′-OH terminus on a semi-preparative reversed phase C18 column (9.4 × 250 mm, Du Pont) using a linear gradient of 18–35% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0 in 45 min at room temperature and 8–20% acetonitrile in 0.1 M triethylammonium acetate, pH 7.0 in 65 min at 55°C, respectively (Chow *et al.*, 1981). The sequence of each primer was confirmed by two-dimensional sequence analysis (Jay *et al.*, 1974) after ^{32}P phosphorylation of the 5′-OH terminus with [γ-^{32}P]ATP and T4 polynucleotide kinase. The labelled primers were freed from excess ATP by chromatography on Sephadex G-50 (fine) using 100 mM triethylammonium bicarbonate buffer (pH 7.6) as eluent.

Reverse transcription of PSTV RNA

Reverse transcription was carried out in five separate batches of 50 μl each containing (in final concentration) Tris-HCl, pH 8.3 (100 mM), dithiothreitol (10 mM), $MgCl_2$ (6 mM), KCl (40 mM), actinomycin D (50 μg/ml), unlabelled deoxyribonucleoside triphosphates (500 μM each) 5′-^{32}P-labelled DNA primer (750 nM) (see legend to Figure 2), AMV reverse transcriptase (1700 units/ml) and partially purified RNA (300 μg/ml) containing ~3 μg/ml PSTV RNA. Prior to cDNA synthesis the RNA sample was taken up in water, heated in the presence of the primer for 2 min at 80°C, and immediately chilled on ice. After incubation for 90 min at 42°C the cDNA samples were combined and the reaction was stopped by the addition of 110 μl 50 mM EDTA, pH 7. The RNA was degraded by alkaline hydrolysis for 30 min at 42°C at a final concentration of 300 mM NaOH in a total volume of 400 μl. The reaction mixture was neutralized with 65 μl 10% HOAc and then loaded on a column (0.9 × 70 cm) of CM-Superose 6B with a wet particle size of 20–40 μm in diameter (provided by Pharmacia) and eluted with a buffer containing Tris-HCl, pH 8.2 (10 mM), EDTA (1 mM) and NaCl (0.2 M). Aliquots from selected fractions were run on an 8% polyacrylamide sequencing gel (200 × 400 × 0.2 mm) which was autoradiographed after drying. Lane RM in Figure 3a represents the total reaction mixture applied to the column, the numbers on top of the subsequent lanes indicate the number of the fractions from which aliquots were taken. The fractions 28–32 containing cDNA in the size range of full-length copies were pooled, ethanol precipitated and used for sequencing.

Sequencing of PSTV cDNA

The PSTV cDNA was sequenced according to the method of Maxam and Gilbert (1980). The products of the four nucleotide-specific chemical cleavage reactions were separated on 8% and 20% polyacrylamide sequencing gels (200 × 400 × 0.2 mm), respectively, and after drying and autoradiography the sequence was read from the autoradiogram. We applied the Maxam-Gilbert technique because the reverse transcriptase pauses at the highly base-paired regions of the PSTV RNA template (see Figure 3a, lane RM) so that the dideoxy-chain-terminating method (Sanger *et al.*, 1977) does not allow us to read certain parts of the sequence on the corresponding gels. Due to the circularity of the PSTV RNA template, a single primer should, in principle, allow the reverse transcription and sequencing of the entire viroid genome. However, all attempts to produce sufficient amounts of longer than full-length transcripts failed. Therefore, it was necessary to use at least two different primers. Thus, overlapping sequences were obtained so that possible mutations present in the primer binding sites also become apparent.

Calculation of secondary structure and thermodynamic properties of the PSTV isolates

The secondary structure of the isolates was calculated on the basis of their nucleotide sequence and their free energy parameters for nucleic acid structures as described and modified by Riesner *et al.* (1979, 1983a, 1983b) and Steger *et al.* (1984). To find the secondary structure with the lowest free-energy at different temperatures a computer program using the algorithm of Zuker and Stiegler (1981), as adapted for circular molecules (Steger *et al.*, 1984), was applied. To evaluate the thermodynamic properties of distinct regions within the PSTV RNA molecule an additional computer program was applied. It had been originally designed to find similar secondary structures in different RNA molecules by comparing regions of interest. This program presents all the secondary structures which could arise from all possible intermolecular base pairing between the nucleotides of two RNA strands. To simulate the actual thermal melting behaviour of both pairing strands of the VM region within the whole molecule, the ends of this region had to be closed in those adjacent helical regions which, according to the results of our secondary structure program, were known to be stable also at higher temperatures. The program computes the free energy values of the various structures and their occupation number. The melting behaviour of the VM region is then evaluated by plotting against temperature the sum of all the equilibrium concentrations of those structures which contain at least all the nucleotides of the upper and lower strand of PM loop 1 in an unpaired form (Figure 4c).

Determination of the sequence homology between viroids

In order to search for sequence homologies between the VM region of PSTV and, in other viroids, a similar region, the total nucleotide sequence of each viroid was first aligned in such a way that a high degree of homology was obtained to the sequence of the upper and lower strands of the VM region of PSTV, respectively. On the basis of the sequence of the viroids of the PSTV group (viroids Nos. 1–5 in Figure 6) and of the HSV group (viroids Nos. 6 and 7 in Figure 6) a consensus sequence was derived according to the method of Lerner *et al.* (1980). In order to prevent in the consensus sequence any nucleotide-predominance which could be caused by such viroids where different isolates are known, we applied the same statistical weight for all viroid species, in that the appearance of a given nucleotide was divided by the total number of the isolates of the corresponding species. A particular nucleotide is represented in the consensus sequence if it appears 45–70% of the time, if it appears 75–94% of the time, it is underlined once; and if it appears 95–100% of the time, it is underlined twice. If different nucleotides are found with the same frequency in the same position they are represented in the consensus sequence by the following symbols: K (GU), S (CG), W (AU), N (any of the four nucleotides). In all those cases where gaps are detected in certain positions in one or more viroids, the capital letters in the consensus sequence for the corresponding nucleotides are replaced by lower case letters. These letters are also used in the different viroid sequences to indicate the difference in the nucleotides as compared with the consensus sequence.

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In a recent paper Visvader and Symons (*Nucleic Acids Res.*, **13**, 2907-2920, 1985) published the sequence of eleven CEV-variants as obtained by molecular cloning from the two new CEV isolates CEV-J and CEV-DE 30. Their chain length was found to vary between 370 and 375 nucleotides. On the basis of their sequence these variants could be grouped into two classes named A and B whose members incite severe and mild disease symptoms in tomato, respectively. The variants of class A are similar to the isolate CEV-A whereas the ones of class B are related to CEV-DE 26. All variants of the new isolate CEV-DE 30 and two out of the nine variants of the new isolate CEV-J belong to class B; the remaining seven CEV-J variants can be grouped into class A.