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Cloned single- and double-stranded DNA copies of potato spindle tuber viroid (PSTV) RNA and co-inoculated subgenomic DNA fragments are infectious

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A set of monomeric and oligomeric potato spindle tuber viroid (PSTV) specific DNA forms representing complete DNA copies of the circular PSTV RNA genome were constructed and cloned in plasmid pBR322 and bacteriophage M13. Both single- and double-stranded PSTV DNAs are capable of initiating viroid replication in mechanically inoculated tomato plants where it normally proceeds *via* the RNA-RNA pathway without DNA being involved. All dimeric and higher multimeric forms were infectious irrespective of their polarity in the case of single-stranded DNA and regardless of their orientation in the vector DNA in the case of double-stranded DNA. The vector-inserted monomeric PSTV DNA units were also found to be infectious but of low specific infectivity which was increased when these monomers had been excised. Even two subgenomic DNA fragments, representing together the 359 nucleotides of the PSTV RNA genome, initiated the synthesis of viroid RNA progeny when co-inoculated although each fragment by itself is non-infectious. These results are discussed with respect to the infectivity previously observed with certain cloned DNAs of conventional RNA and DNA viruses.

Key words: infectious viroid cDNA/ligation *in vivo*/recombinant DNA/viroid cloning

Introduction

Viroids are the smallest and structurally best characterized disease agents known. They represent unencapsidated single-stranded covalently closed RNA molecules with a chain length of ~240 – 380 nucleotides, depending on the viroid 'species'. Thus far, viroids have only been found in higher plants where they cause several economically important diseases. The primary structure of several different viroid 'species' and detailed biochemical and biophysical studies show that all known viroids follow a common principle of structure and dynamics. Viroids exist in their native state as unbranched rod-like structures in which short double helices alternate with small internal loops. Despite the wealth of information on the molecular and structural features of viroids, little is known about their biological functions; this is related to experimental difficulties inherent in plant systems. As a result the possible mode of interaction of viroids with the host cell, and especially the mechanism of viroid replication and pathogenesis, has given rise to much speculation (for review, see Sänger, 1982, 1984).

Our sequencing studies on different potato spindle tuber viroid (PSTV) field isolates revealed that their virulence for tomato plants can be correlated with nucleotide changes in a distinct region of the PSTV molecule, the so-called 'virulence modulating (VM) region' (Schnölzer *et al.*, in preparation). This finding provides the basis for studying viroid pathogenicity experimentally by introducing site-specific mutations into the VM region of cloned single-stranded PSTV cDNA with the aid of mismatch priming. One precondition for this experimental approach would be that cloned viroid-specific DNA is infectious, which is indeed the case as recently found by Cress *et al.* (1983) for PSTV and by Meshi *et al.* (1984) for hop stunt viroid (HSV).

Here we report that not only the plasmid cloned double-stranded DNA, but also the phage M13-cloned single-stranded PSTV DNAs of both polarities are infectious for tomato plants where they induce the synthesis of normal PSTV (+) RNA progeny. The biological effects resulting from site-specific mutagenesis can now be studied in a straight-forward way by inoculating this altered DNA into tomato host plants and subsequently analysing the ensuing biological changes in viroid replication, host specificity and in the development and severity of disease.

Results

Subcloning of the complete PSTV genome into bacteriophage M13 and plasmid pBR322

We have constructed the plasmid pMT40 containing a *Bam*HI insert of 359 bp representing a correct DNA copy of the entire genome of the severe PSTV field isolate KF440

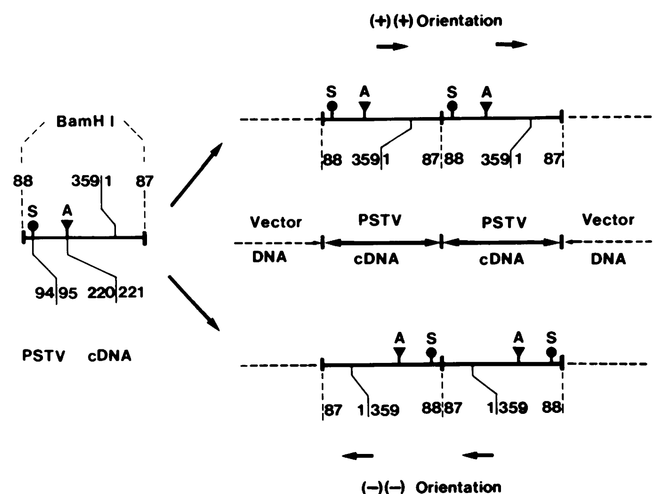

Fig. 1. Orientation of insertion and connection of PSTV DNA molecules within the vector DNA. The **left part** of the figure symbolizes a double-stranded *Bam*HI unit of PSTV DNA which comprises a full-length copy of the circular PSTV RNA molecule with its 359 nucleotides. Based on the arbitrarily defined numbering system introduced by Gross *et al.* (1978) nucleotide 88 represents the 5′ and nucleotide 87 the 3′ terminus of the (+) strand of the linear *Bam*HI construct of PSTV DNA. In addition, the restriction sites *Sma*I (S) and *Ava*II (A) and their relative position to the conventional start and end point of numbering of the originally circular PSTV RNA molecule are given. The **right part** depicts the two possible modes of insertion of two head-to-tail connected *Bam*HI units of PSTV DNA into a vector DNA. On the basis of this definition the upper recombinant PSTV construct represents a (+ +)-oriented and the lower one a (− −)-oriented clone.

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Table I. Infectivity of double-stranded vector DNA containing different PSTV DNA inserts

Test No.	Double-stranded DNA		Orientation of insertion	Treatment	μg DNA/plant at dilution 10^0	Infectivity index[a] at dilution			
	Type of vector	PSTV insert				10^0	10^{-1}	10^{-2}	10^{-3}
1.	pBR322	1 x *Bam*HI	+	None	45	1	0	–	–
2.	pBR322	1 x *Bam*HI		*Bam*HI	15	8	–	–	–
3.	pBR322	2 x *Bam*HI	– –	None	48	10	9	0	0
4.	pBR322	2 x *Bam*HI	– –	RNase	48	10	7	–	–
5.	pBR322	2 x *Bam*HI	– –	DNase	48	0	–	–	–
6.	pBR322	None		None	48	0	–	–	–
7.	M13 mp11	1 x *Bam*HI	+	None	34	4	–	–	–
8.	M13 mp11	1 x *Bam*HI	–	None	34	1	–	–	–
9.	M13 mp11	4 x *Bam*HI	+ + + +	None	68	10	10	3	1
10.	None	4 x *Bam*HI[b]	+ + + +	Nuclease S1	3	10	10	7	3
11.	M13 mp8	1 x *Ava*II	+	None	68	1	0	–	–
12.	M13 mp8	1 x *Ava*II		*Ava*II	17	9	7	–	–
13.	None	1 x *Ava*II	+	RNase	2.4	10	6	–	–
14.	M13 mp8	3 x *Ava*II	+ + +	None	68	10	10	10	1
15.	M13 mp8	None		None	68	0	–	–	–

[a]Total number of symptom-bearing, PSTV-containing tomato plants out of 10 plants inoculated by leaf rubbing; – not tested.
[b]This fragment was obtained by *Hind*III/*Eco*RI digestion of the M13 mp11 clone (No. 9) containing 4 *Bam*HI units of PSTV DNA.

(Tabler *et al.*, in preparation). The *Bam*HI unit of pMT40 was cloned into the *Bam*HI site of bacteriophage M13 mp11 in both orientations. To distinguish between these two orientations we applied the same numbering system to the PSTV DNA which we have previously used for the PSTV RNA (Gross *et al.*, 1978). Thus the DNA strand corresponding to the PSTV (+) RNA is called the (+) strand whereas the complementary strand is called the (–) strand. We define the (+) orientation of insertion as the connection of the (+) strand of PSTV DNA with that strand of the vector DNA which is numbered from the 5′ to the 3′ end by convention. It follows that a (+)-oriented *Bam*HI insert of the PSTV DNA copy will have its *Sma*I site at the left end of the defined map of the recombinant vector (see Figure 1, top). Thus, a (+)-oriented insertion connects the PSTV (+) DNA with the bacteriophage (+) DNA strand of the double-stranded replicative form of M13. Since the mature bacteriophage particles contain only the M13 (+) DNA strand the inserted (+) PSTV DNA strand becomes amplified upon M13 propagation. For amplifying single-stranded (–) PSTV DNA, a (–) oriented insertion of the double-stranded PSTV DNA is required.

To obtain head-to-tail connected tandem molecules, the isolated 359-bp *Bam*HI fragment of pMT40 was subcloned into pBR322. Restriction analysis showed that clone pMT41 contains a (– –) dimer (Figure 1, bottom). After incomplete *Bam*HI digestion this dimeric fragment was also subcloned into the *Bam*HI site of M13 mp11. Among the eight recombinant phages tested, unexpectedly only (+ +)-oriented clones were identified, one of which showed (+ + + +)-orientation. The corresponding (– –) and (– – – –) clones were constructed by turn-around-cloning of the *Hind*III-*Eco*RI fragment of the (+)-oriented dimeric and tetrameric clones into the bacteriophage M13 mp10. In addition, pMT41 containing a head-to-tail connected PSTV DNA dimer was digested with *Ava*II whereupon a 359-bp *Ava*II fragment is released which was subcloned into the unique *Ava*II site of bacteriophage M13 mp8. Three types of recombinant phages were identified containing one, two or three *Ava*II units of PSTV DNA. Since PSTV DNA and M13 DNA share the same non-palindromic *Ava*II restriction site (G′GACC) only (+)-oriented clones can be obtained. As the final result of our cloning work a set of double- and single-stranded PSTV DNAs was available, whose infectivity for the PSTV host plant tomato was tested.

Infectivity of cloned double-stranded PSTV DNA units

DNA was isolated from the various PSTV clones and the different samples were mechanically inoculated by rubbing them into the carborundum-dusted leaves of at least 10 tomato seedlings, as with PSTV RNA. From all the DNAs expected to be possibly infectious, several 10-fold diluted solutions were bioassayed in addition. Two to three weeks after inoculation the first foliar symptoms of a severe PSTV infection appeared; epinasty, malformation and veinal necrosis of leaves, followed by growth retardation and stunting of the total plant.

The results of the bioassay of the double-stranded PSTV DNAs are summarized in Table I. The different isolated forms of vector DNA containing monomeric or higher forms of double-stranded PSTV DNA were leaf-inoculated directly onto tomato plants. Aliquots of these samples were treated with restriction enzymes or DNase and RNase prior to inoculation. The dimeric and higher multimeric forms of plasmid pBR322- and bacteriophage M13-cloned PSTV DNA were infectious irrespective of whether the viroid DNA used for inoculation was inserted in the complete vector DNA (Table I, No. 3, 9, 14) or had been excised with the appropriate restriction enzymes (Table I, No. 10). The fragment consisting of four monomeric PSTV units plus short flanking regions derived from the polylinker of the vector DNA used in this latter case was obtained by mixed digestion of the M13-cloned *Bam*HI PSTV DNA with *Hind*III and *Eco*RI.

The recombinant DNA of four clones containing either the monomeric *Bam*HI or the monomeric *Ava*II PSTV DNA unit still inserted on the pBR322 or the M13 vector proved also to be infectious but the infectivity index was relatively low (Table I, No. 1, 8 and 11). However, the monomeric (+)-oriented *Bam*HI unit in M13 mp11 (Table I, No. 7) was significantly more infectious than the others. The infectivity of these samples increased greatly when the monomeric PSTV DNA inserts were excised from the vector (compare Table I No. 1 with No. 2 and No. 11 with No. 12 and 13). The infectivity of the excised *Ava*II monomer was not influenced

if it had been inoculated together with the vector DNA (Table I, No. 12) or purified by chromatography (Table I, No. 13).

To ascertain whether the infectious entity was really a DNA and not a minute contamination of viroid RNA, a sample was treated with RNase, and part of it in addition with RNase-free DNase. The RNase treatment did not significantly affect the infectivity of the DNA probe (Table I, No.3 and 4) whereas exposure to DNase destroyed infectivity (Table I, No. 5). Nuclease S1 treatment did not destroy infectivity as expected for the double-stranded nature of the tetrameric *Bam*HI unit of PSTV DNA (Table I, No. 10). Control plants inoculated with the DNA of the vectors alone developed no symptoms of PSTV infection (Table I, No. 6 and 15).

In vivo ligation between non-infectious double-stranded subgenomic PSTV DNA fragments

The high infectivity index of the excised double-stranded PSTV DNA monomers described in the previous section is most probably due to intracellular ligation of these monomers to oligomeric DNAs after inoculation. One way to verify whether this type of ligation of DNA molecules is actually operating is the infectivity assay of subgenomic and hence non-infectious PSTV DNA fragments. Provided that these fragments can in principle be ligated to full-length PSTV-DNA copies (and oligomers thereof) mixed inoculation of such fragments should lead to viroid infection.

PSTV DNA has two *Ava*I sites on its circular restriction map so that a small and a large *Ava*I fragment with 167 and 192 bp can be obtained. We isolated these fragments from recombinant plasmids, each of which contained only a part of the PSTV genome as described (Tabler *et al.*, in preparation). When these two subgenomic *Ava*I fragments are ligated *in vivo* in such a way that the entire PSTV genome with the correct linear array of its 359 nucleotides is formed, they should be able to initiate PSTV replication. The results of the bioassay show that the two *Ava*I fragments were not infectious if inoculated separately (Table II, No. 1 and 2) whereas the mixed inoculations of both fragments resulted in the infection of nine out of 20 test plants (Table II, No. 3) substantiating that DNA ligation must occur *in vivo*.

Infectivity of single-stranded PSTV DNAs

Table III shows the results of the infectivity tests of the various forms of M13-cloned single-stranded PSTV DNAs. Again all dimeric and higher multimeric forms of single-stranded PSTV DNA proved to be infectious irrespective of whether the strand of (+) or (−) polarity was used for inoculation (Table III, No. 3−5, 9, 12 and 13). Control experiments with DNase, RNase and single-strand-specific nuclease S1 were performed to ascertain that the infectivity of the samples was actually based on the single-stranded DNA and not due to minute amounts of contaminating RNA or double-stranded DNA, whose presence in these preparations became evident only when excessive amounts were analysed on agarose gels. RNase treatment had no effect on the infectivity (Table III, No. 5, 6 and 9, 10) indicating that the infectious entity is DNA. This was further confirmed by DNase treatment of the sample whereupon none of the 10 inoculated plants developed PSTV symptoms (Table III, No. 7). The digestion with nuclease S1 destroyed infectivity completely (Table III, No. 8) substantiating that the infectious entity in these preparations is, in fact, single-stranded DNA.

In addition to the dimeric and higher multimeric forms, we

Table II. Infectivity test of double-stranded PSTV DNA subfragments inoculated separately and as a mixture

Test No.	PSTV DNA subfragment	Nucleotide number of the (+) strand (5′→3′ terminus)	Length in nucleotides	μg DNA inoculated/plant	Infectivity index[a]
1.	*Sma*I *Ava*I[b]	285-(359/1)-92	167	1	0
2.	Large *Ava*I[b]	93−284	192	1	0
3.	Small *Ava*I[b] + Large *Ava*I[b]	285-(359/1)-92 + 93−284	167 + 192	1 + 1	9

[a]Total number of symptom-bearing PSTV-containing tomato plants out of 20 plants inoculated by leaf-rubbing.
[b]PSTV DNA with its 359 nucleotides has two *Ava*I sites at position 92/93 and 284/285 on its circular restriction map so that two different subgenomic *Ava*I fragments can be obtained.

Table III. Infectivity of single-stranded M13 DNA containing different PSTV inserts

Test No.	Single-stranded DNA: Type of M13 vector	Single-stranded DNA: PSTV insert	Polarity[a]	Treatment	μg DNA/plant at dilution 10^0	Infectivity index[b] at dilution 10^0	10^{-1}	10^{-2}
1.	mp11	1 x *Bam*HI	+	None	272	8	−	−
2.	mp11	1 x *Bam*HI	−	None	133	0	−	−
3.	mp11	2 x *Bam*HI	+	None	272	10	9	0
4.	mp10	2 x *Bam*HI	−	None	7	5	1	0
5.	mp11	4 x *Bam*HI	+	None	62	10	8	0
6.	mp11	4 x *Bam*HI	+	RNase	62	10	9	−
7.	mp11	4 x *Bam*HI	+	DNase	62	0	−	−
8.	mp11	4 x *Bam*HI	+	Nuclease S1	62	0	−	−
9.	mp10	4 x *Bam*HI	−	None	7	9	5	−
10.	mp11	4 x *Bam*HI	−	RNase	7	10	5	−
11.	mp8	1 x *Ava*II	+	None	140	1	−	−
12.	mp8	2 x *Ava*II	+	None	62	10	7	0
13.	mp8	3 x *Ava*II	+	None	7	8	5	0
14.	mp8	None		None	10	0	−	−
15.	mp10	None		None	110	0	−	−
16.	mp11	None		None	10	0	−	−

[a]The circular PSTV RNA accumulating in plants is arbitrarily defined to be of (+) polarity.
[b]Total number of symptom-bearing, PSTV-containing tomato plants out of 10 plants inoculated by leaf rubbing; − not tested.

Table IV. Infectivity of nucleic acid extracts from an *E. coli* culture harbouring a pBR322 derivative containing four (+) oriented head-to-tail connected *Bam*HI units of PSTV DNA

Test No.	Type of nucleic acid	Treatment	Amount inoculated/plant at dilution 10^0	Infectivity index[a] at dilution			
				10^0	10^{-1}	10^{-2}	10^{-3}
1.	Total RNA[b]	None	150 μg	10	10	10	7
2.	Total RNA[b]	DNase	150 μg	10	10	10	6
3.	Total RNA[b]	RNase	150 μg	0	–	–	–
4.	Total nucleic acids[c]	None	100 μg[d]	10	10	1	–

[a]Total number of symptom-bearing, PSTV-containing tomato plants out of 10 plants inoculated by leaf rubbing; – not tested.
[b]The RNA moiety present in a nucleic acid preparation obtained during plasmid isolation was separated from the plasmid DNA by chromatography on a Sephacryl S1000 column.
[c]The total nucleic acids were extracted from a stationary *E. coli* culture with the aid of phenol, ethanol precipitated, redissolved and then used for inoculation.
[d]Equivalent of 2 ml stationary culture.

also bioassayed three monomeric single-stranded PSTV DNAs. When 140 μg of the single-stranded M13 DNA containing the monomeric (+) *Ava*II strand of PSTV DNA were inoculated per tomato seedling only one out of 10 plants developed PSTV symptoms (Table III, No. 11). The single-stranded *Bam*HI monomer of (–) polarity was non-infectious whereas the corresponding (+) form was capable of inducing PSTV symptoms in tomato (Table III, No. 1 and 2).

The results of all the infectivity assays with the different cloned PSTV DNA forms are summarized in Table V.

Presence of infectious PSTV RNA in Escherichia coli cells

To test whether or not *E. coli* cells which harbour a pBR322 plasmid containing four head-to-tail connected *Bam*HI units of PSTV DNA in (+) orientation also produce infectious PSTV RNA progeny, we purified and bioassayed the RNA moiety from a nucleic acid preparation obtained during plasmid preparation. In parallel, a total nucleic acid extract of the same *E. coli* cells was prepared by direct phenol extraction of a 30 ml culture. Equivalents of 2 ml culture and two dilutions of this sample were also inoculated onto tomato plants. Table IV shows that the RNA fraction is in fact highly infectious (Table IV, No. 1). The infectivity of this sample was resistant to DNase but completely destroyed by RNase treatment (Table IV, No. 2 and 3) substantiating that no contaminating infectious DNA was present in this crude RNA preparation and that the PSTV DNA insert present in the plasmid pBR322 must have been transcribed into RNA by *E. coli* RNA polymerase. The high infectivity of the total nucleic acid extract from the viroid DNA-containing *E. coli* clone facilitates biological screening since crude preparations can be used routinely to directly bioassay large numbers of clones for their ability to induce viroid infection.

Absence of PSTV DNA and presence of PSTV RNA progeny in tomato plants inoculated with cloned PSTV DNA

To detect viroid-specific DNA sequences possibly synthesized in tomatoes inoculated with PSTV DNA, one plant representative for each inoculum was chosen from the infectivity trial after disease symptoms had appeared in the positive cases of the assay. The total nucleic acids were isolated from the leaves of these plants and analysed by molecular hybridisation on Northern blots with ^{32}P-labeled (+) or (–) PSTV-specific recombinant M13 DNA under conditions stringent for detecting only (–) or (+) PSTV sequences, respectively (Spiesmacher *et al.*, in preparation). The total nucleic acids from all plants which showed the characteristic PSTV disease symptoms gave an identical hybridisation pattern with either probe regardless of whether the plants had been inoculated with cloned viroid-specific single- or double-stranded DNA or with natural viroid RNA. As in the case of a normal PSTV infection, in all these plants predominantly monomeric circular and some linear PSTV (+) RNA was found (Figure 2) and only traces of PSTV (–) RNA molecules (data not shown) could be detected.

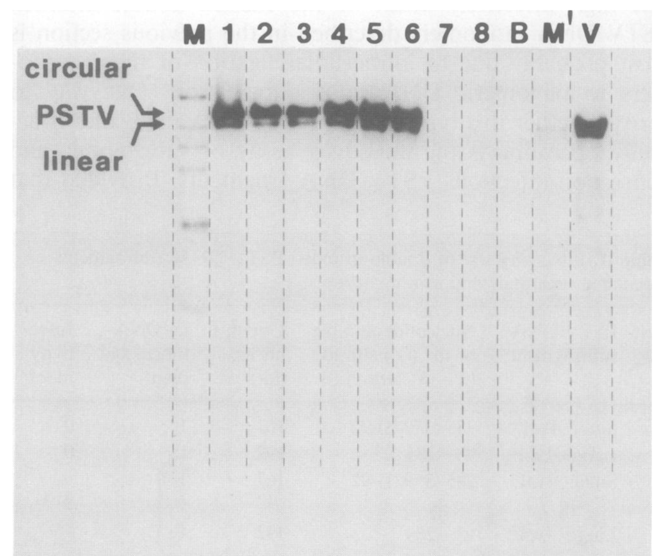

Fig. 2. Northern blot analysis of total nucleic acid extracts from tomato plants inoculated with single-stranded bacteriophage M13 DNA containing different PSTV-specific DNAs of (+) and (–) polarity. Phenol-extracted nucleic acids were glyoxalated and separated on a 5% polyacrylamide gel. After electro-transfer to Gene Screen filter the blot was hybridised to a ^{32}P-labeled M13 probe specific for PSTV-RNA and PSTV-DNA of (+) polarity. The lanes on the gel were loaded with nucleic acid extracts from plants inoculated with different single-stranded M13 DNA containing the following head-to-tail connected PSTV – DNA units of full length; their (+) or (–) polarity and their ability to induce PSTV symptoms (S) or no symptoms (NS) in the inoculated tomato plants is indicated. **Lane 1**, 4 x *Bam*HI (+) units, (S). **Lane 2**, 2 x *Bam*HI (+) units, (S). **Lane 3**, 2 x *Ava*II (+) units, (S). **Lane 4**, 3 x *Ava*II (+) units, (S). **Lane 5**, 4 x *Bam*HI (–) units, (S). **Lane 6**, 2 x *Bam*HI (–) units, (S). **Lane 7**, M13 mp10 control sample, (NS). **Lane 8**, M13 mp11 control sample, (NS). **Lane B**, buffer-inoculated healthy control sample, (NS). **Lane V**, PSTV-RNA inoculated control sample (S). **Lane M**, ^{32}P-labeled *Hinf*I fragments of pBR322 as size marker. **Lane M'**, ^{125}I-labeled PSTV-RNA as size marker. As compared with the PSTV-RNA control infection **(Lane V)** in all other symptom-bearing plants **(Lanes 1–6)** an identical signal based on the presence of circular and linear PSTV-RNA progeny is observed. This finding substantiates that irrespective of its type, size and polarity the M13-cloned and still vector-inserted single-stranded PSTV-specific dimeric and multimeric DNA is capable of inducing PSTV RNA replication and PSTV-specific disease symptoms in tomato plants.

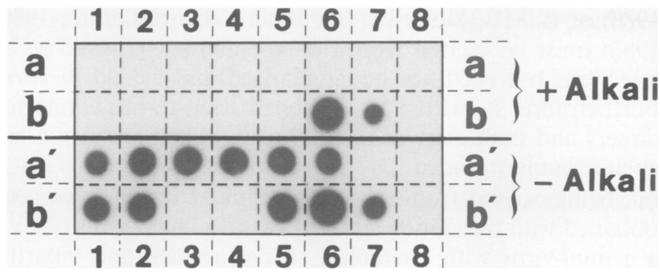

Fig. 3. Dot spot analysis of total nucleic acid extracts to probe for the presence of PSTV-specific DNA which could have survived and/or become replicated after inoculation of tomato plants with various single-stranded **(rows a and a′)** and double-stranded **(rows b and b′)** PSTV-specific DNAs. Alkali-treated **(rows a and b)** and untreated **(rows a′ and b′)** nucleic acids equivalent to 10 mg of leaf material were glyoxalated and spotted on Gene-Screen filter in corresponding positions and hybridised to a ^{32}P-labeled M13 DNA probe, specific for (+) and (−) PSTV sequences. The samples spotted in **row a** and **a′**, **fields 1−8** correspond to the ones analysed in Figure 2 lanes 1−8. In **row b** and **b′** extracts were spotted from plants which had been inoculated with double-stranded M13 DNA containing four *Bam*HI of PSTV-DNA **(b1, b′1)**, with a pBR322 derivative containing two **(b2,b′2)** or one **(b3,b′3)** *Bam*HI unit(s). In **position b4** and **position b′4** the extract from the buffer-inoculated healthy control plants was spotted. **Positions b5** and **b′5** contained the extract from plants inoculated with PSTV-RNA. As an additional control 500, 15 and 0.5 ng of a purified plasmid containing one PSTV-DNA unit were treated and spotted in the same way to **position b6−b8** and **b′6−b′8**, respectively. All the untreated nucleic acid samples from symptom-bearing plants gave a strong hybridisation signal which was absent in the alkali-treated aliquot in which the RNA had been degraded. However, the hybridisation signal of the purified plasmid, containing PSTV-specific DNA sequences was not affected by alkali treatment. This result substantiates that only viroid-specific RNA but no viroid-specific DNA is present in the symptom-bearing plants.

Table V. Summarized results of the infectivity tests of cloned PSTV DNAs as obtained by bioassays on tomato plants

Type of cloned PSTV-specific DNA			Infectivity
Single-stranded	Monomeric form	(+) polarity	+ and + +
		(−) polarity	−
	Oligomeric form	(+) polarity	+ + +
		(−) polarity	+ + +
Double-stranded	Monomeric form	Inserted in vector	+
		Excised from vector	+ + +
	Oligomeric form	Inserted in vector	+ + +
		Excised from vector	+ + +
	Subfragments inoculated separately		−
	Subfragments ligatable to a monomer, co-inoculated		+

−: no infectivity observed.
+, + +, + + +: low, intermediate, high specific infectivity, respectively.

To ensure that the hybridisation signals were due to viroid RNA and not to viroid-specific DNA progeny, dot-spot hybridisation experiments were performed in which the nucleic acids from the tomato leaves were alkaline-treated to degrade the RNA and to denature the remaining DNA into its single-stranded form. As a control the untreated samples of these nucleic acids were used. In addition plasmid pMT40 DNA containing a monomeric *Bam*HI unit of PSTV DNA was treated in the same way to ensure that the hybridisation signal of DNA is not affected by the alkaline treatment applied.

The hybridisation signal disappeared after alkaline treatment of the nucleic acids extracted from the inoculated plants whereas the signal of the PSTV DNA containing pMT40 plasmid DNA was not affected (Figure 3). This shows that in the tomato plants infected with the cloned PSTV DNA only PSTV-specific RNA and no viroid DNA accumulates. One must conclude, therefore, that the cloned PSTV DNAs taken for inoculation are not replicated as such in the infected plants. It follows that all of the cloned and infectious PSTV DNAs must be capable of inducing a normal PSTV replication process by initiating the synthesis of PSTV (+) and/or (−) RNA molecules. As a result a normal viroid replication process ensues during which all the putative intermediate forms of PSTV replication occur and the circular PSTV (+) RNA molecules finally accumulate.

Discussion

Recombinant double-stranded DNAs containing head-to-tail-connected PSTV DNA dimers are infectious and capable of inducing the tomato seedlings (Cress *et al.*, 1983), as are recombinant DNAs of hop stunt viroid (Meshi *et al.*, 1984). As summarized in a synoptic way in Table V, our present data confirm theirs and extend them in three aspects of general interest. Firstly, we found that not only double-stranded but also all M13-cloned dimeric and multimeric single-stranded PSTV DNAs are infectious irrespective of whether they represent the PSTV (+) or (−) DNA strand. Secondly, certain cloned monomeric single-stranded as well as double-stranded PSTV DNAs are also infectious. Thirdly, subgenomic fragments of cloned PSTV DNA, which are non-infectious themselves, are capable of initiating infection when inoculated as a mixture. These infecting PSTV-specific DNAs are not replicated as such in the tomato plants but, as with a normal viroid infection, predominantly circular and some linear PSTV RNA molecules of unit length accumulate. Although it is not known how these DNAs initiate viroid replication, the presence of normal PSTV RNA progeny proves that the DNA must have served as template for PSTV RNA synthesis. Which of the three nuclear DNA-dependent RNA polymerases of the host plant accepts and transcribes the cloned PSTV-specific DNAs is unknown. Evidently, multimeric PSTV RNA transcripts are formed which are finally processed to circular PSTV RNA molecules of unit length. This is of particular interest because viroid replication proceeds normally *via* the RNA-RNA pathway involving multimeric replicative intermediates of (−) and (+) polarity (Rohde and Sänger, 1981; Branch *et al.*, 1981; Owens and Diener, 1982; Mühlbach *et al.*, 1983; Spiesmacher *et al.*, 1983, and in preparation) and neither in healthy nor in PSTV-infected tomato tissue could any PSTV-specific DNA sequences be detected (Branch and Dickson, 1980; Zaitlin *et al.*, 1980; Rohde and Sänger, 1981; Hadidi *et al.*, 1981).

Infectivity of cloned double-stranded PSTV DNA

The ability of cloned double-stranded viroid DNA to initiate the synthesis of single-stranded viroid RNA progeny resembles that of the cloned double-stranded cDNA of the single-stranded RNA genome of bacteriophage Qβ (Taniguchi *et al.*, 1978) and poliovirus (Racaniello and Baltimore, 1981). A somewhat different situation exists in the case of cloned and infectious proviral DNA of the linear RNA genome of the retroviruses (e.g., O'Rear *et al.*, 1980; Scott *et al.*, 1981; Kim *et al.*, 1982), because in contrast to phage Qβ, poliovirus and PSTV, a DNA copy is involved in the normal

life cycle of these retroviruses.

The infectivity of the cloned double-stranded PSTV DNA suggests that it is accepted as a DNA template by at least one of the host's three nuclear DNA-dependent RNA polymerases. Our observation that all dimeric or higher forms of head-to-tail connected double-stranded PSTV DNA units are infectious is quite plausible since these DNA forms allow the transcription into dimeric or higher multimeric (+) or (−) viroid RNA which are probably identical to the naturally occurring intermediates of viroid replication; the latter occur in the nucleus of the host cell and from them the viroid RNA progeny can finally become transcribed and/or processed (Spiesmacher *et al.*, 1983, and in preparation). The cloned monomeric *Ava*II and *Bam*HI units of PSTV DNAs have a comparatively low infectivity index if still inserted in the vector (Table I, No. 1, 7, 8 and 11). This is at variance with the results of Cress *et al.* (1983) whose corresponding clones were reported to be non-infectious, perhaps because in their bioassay only one to four plants were used. The low infectivity of the vector-inserted monomeric *Ava*II and *Bam*HI units was greatly increased after excision from the vector DNA (Table I, No. 2 and 12). This increase in infectivity can be explained by assuming that only the excised monomeric PSTV DNA units can be ligated in the host cells to higher forms prior to their transcription into multimeric RNA intermediates. This interpretation is supported by our finding that mixed inoculations with the two non-infectious subgenomic *Ava*I PSTV DNA fragments lead to infection and to the synthesis of normal PSTV RNA progeny. Presumably these fragments, when inoculated together, are ligated *in vivo* to a complete and hence infectious PSTV DNA (and possibly to oligomers thereof). Such a ligation step in which two separate subgenomic fragments become an integral PSTV genome could, in principle, also operate at the RNA level. However, apart from the complexity of such a multi-step process, the existence of appropriate restriction termini in particular predisposes the DNA fragments to become ligated in the correct fashion. *In vivo* DNA ligation has already been proposed to explain how cloned non-infectious DNA fragments of the cauliflower mosaic virus (CaMV) genome regain infectivity when inoculated simultaneously (Lebeurier *et al.*, 1982).

The low infectivity of the vector-inserted, and hence not ligatable, monomeric *Ava*II and *Bam*HI units of PSTV DNA can be explained by assuming that it resembles artificially and randomly linearized PSTV RNA molecules, which are also much less infectious than the viroid RNA circles (Sänger *et al.*, 1979) and the corresponding 'natural' linear forms with their defined termini.

Details of how PSTV DNA is transcribed into (+) or (−) PSTV RNA have yet to be delineated. In the case of the viroid DNA still inserted in the vector DNA, transcription could start inside the procaryotic vector sequences and then proceed through the viroid DNA or it could also begin at the PSTV DNA insert itself. Our experiments with the excised monomeric *Ava*II and *Bam*HI units suggest that the PSTV DNA itself is able to promote its transcription into RNA.

The fact that excised double-stranded PSTV DNA monomers are infectious while the same monomers inserted in the vector have low specific infectivity is reminiscent of the behaviour of the cloned double-stranded DNAs of certain conventional DNA viruses which are characterized, like the PSTV RNA genome, by the circularity of their double-stranded or single-stranded DNA genome. As with PSTV, the cloned double-stranded monomeric viral DNA of polyoma virus (Chan *et al.*, 1979; Fried *et al.*, 1979), SV40 (Schaffner, 1980) or CaMV (Howell *et al.*, 1980; Lebeurier *et al.*, 1980, 1982) must be excised from the recombinant plasmid to be infectious but need not be circularized and ligated *in vitro*. Furthermore, as with PSTV, cloned head-to-tail connected dimers and multimers of these viruses, which all have a circular double-stranded DNA genome, are infectious without being excised from the vector. Similar results have been obtained with the cloned DNA of cassava latent virus (CLV), a gemini-virus with a circular, single-stranded and bipartite DNA. Upon excision from the recombinant vectors the two linear double-stranded DNAs together are infectious in *Nicotiana benthamiana* where they induce the synthesis of CLV with its two single-stranded circular DNAs (Stanley, 1983). Provided the cloned and excised linear DNA monomers are ligated to circular monomers the replication of all these DNA viruses can proceed by exactly the same mechanism operating after infection with their conventional virions. However, in contrast to all these viruses, neither healthy nor PSTV-infected host (tomato) tissue contains any PSTV-specific DNA sequences, and PSTV RNA replication is known to normally proceed *via* the RNA-RNA pathway.

Infectivity of cloned single-stranded PSTV DNA

Cloned single-stranded PSTV DNAs of (+) and of (−) polarity are capable of inducing PSTV RNA replication and disease symptoms in tomato plants. To our knowledge, this is the first example where a recombinant single-stranded DNA of a pathogen is infectious. This finding is rather surprising since single-stranded DNA, which is normally not present in eucaryotic cells, is nevertheless able to give rise to a replicable pathogenic RNA genome. The only exceptions to the rule that natural DNA is duplex are certain small viruses whose DNA genome is a single-stranded circular molecule. The replication of these viruses, including the gemini viruses, depends on the replication functions of the host which convert the single-stranded viral DNA to a double-stranded molecule, which serves as a template for transcription. One could assume that the infectious circular single-stranded M13 DNA containing one to four units of PSTV DNA is made double-stranded after it has entered the tomato leaf cells. However, since this single-stranded DNA is also accepted by the DNA-dependent RNA polymerases I, II and III *in vitro* (unpublished results) the possibility exists that the cloned single-stranded PSTV DNA is directly transcribed into PSTV RNA. We cannot distinguish at present which of these two modes of transcription is actually responsible for the infectivity of the M13-cloned single-stranded PSTV DNAs of (+) or (−) polarity.

The interpretation of the infectivity of the M13-cloned single-stranded PSTV DNA is complicated by the finding that the single-stranded monomeric *Bam*HI unit of (+) polarity is highly infectious whereas the corresponding (−) form is not. One should expect, therefore, that the cloned double-stranded *Bam*HI monomer would be infectious even if still inserted in its vector because it contains the potentially infectious *Bam*HI (+) DNA strand. However, the double-stranded monomeric *Bam*HI PSTV DNA unit cloned into pBR322 or into M13 mp11 in (−) orientation had a very low specific infectivity. This is in accordance with the situation in all conventional viruses with a circular (DNA) genome (see above). On the other hand the double-stranded DNA of the M13 mp11 construct carrying a (+) *Bam*HI unit of PSTV DNA had a significantly higher infectivity index.

The obvious discrepancy between the results of the infectivity assays obtained with M13-inserted *Bam*HI clones depending on the polarity of single-stranded DNA and on the orientation of insertion of the double-stranded DNA led us to analyse the sequences adjacent to the junction between the M13 vector DNA and PSTV DNA. In the mp11 strain of bacteriophage M13 the *Sma*I site is positioned at the right side of the *Bam*HI site. Coincidently, the sequence of 11 nucleotides (GGATCCCCGGG), which creates the two adjacent *Bam*HI and *Sma*I restriction sites in the polylinker of the mp11 strain of bacteriophage M13, is also present in the PSTV sequence. Consequently a *Bam*HI monomer which is inserted into the *Bam*HI site of M13 mp11 in (+) orientation results in a clone which possesses this segment of 11 nucleotides twice. Therefore, this recombinant clone consists of the complete 359 nucleotides of the monomeric PSTV sequence plus 11 PSTV-specific nucleotides which originate from the vector. The (−) orientation of insertion of the *Bam*HI unit, or the insertion in both possible orientations into the *Bam*HI site of plasmid pBR322, however, will only lead to a clone consisting of 359 plus six PSTV-specific nucleotides originating from the vector. This difference of five nucleotides seems to be essential for the infectivity of the cloned PSTV-DNA. Interestingly, the region in question is part of the central region which is strictly conserved in all viroids sequenced so far, with the exception of avocado sunblotch viroid. In fact, because of this strict conservation, we have previously surmised that this central region is essential for viroid replication (see Sänger, 1982). Site-specific mutations which are presently underway in our laboratory will hopefully help to determine the importance of individual nucleotides in this region for the replication and subsequent processing of the PSTV RNA molecule.

The availability of infectious DNA copies, especially those in M13 vectors, allows the alteration of the primary structure of PSTV by site-specific mutations at the DNA level. In addition, cloned DNA of different viroid isolates and 'species' can be used for the construction of chimeric viroid molecules. Thus mutagenesis and recombination will help to unravel the relationship between chain length, nucleotide sequence and secondary structure of viroids and their replicability, pathogenicity, and host-specificity. It may even be possible to construct effective non-virulent strains of viroids incapable of reverting to virulence and use them to cross-protect crop plants against infections with virulent strains.

Infectious viroid-specific DNA may also become a versatile tool in plant molecular biology. It is capable of initiating RNA transcription and has thus the potential to serve as a promoter. The RNA transcripts themselves are amplified by replication, invade the plant systemically and can easily be detected. Therefore, viroid-specific DNA might be a promising marker for transformation and/or transcription, especially if it is combined with appropriate vector systems for higher plants such as the Ti-plasmid. It is even conceivable that such a vector can be developed from the viroid-specific DNA itself.

Materials and methods

General methods

All digestions with restriction enzymes obtained from Bethesda Research Labs., New England Biolabs and Renner (Dannstadt) were carried out according to the instructions of the manufacturers. Digestions with RNase A (Boehringer, Mannheim), RNase-free DNase I (Renner), transformation into *E. coli* strains *5K, JM103* ligation with T4 DNA ligase (Renner) and recovering DNA fragments from agarose gels were carried out as described in the laboratory manual of Maniatis *et al.* (1982).

Cloning into bacteriophage M13

The cloning into bacteriophage M13, its cultivation in *E. coli* strain *JM103*, the isolation of single-stranded DNA, and the synthesis of the complementary DNA strand initiated by the sequencing primer (Boehringer, Mannheim) was performed as decribed by Messing (1983).

Isolation of double-stranded M13 and plasmid DNA

Double-stranded M13 DNA was extracted from infected *E. coli* cells in the same way as plasmid DNA from chloramphenicol-amplified cultures (Friedrich, 1980) and purified by chromatography with Sephacryl S1000 (Pharmacia Fine Chemicals) as described by Müller and Kütemeier (1982). The latter method was used also for isolation of DNA fragments or total *E. coli* RNA.

Extraction of total nucleic acids from tomato leaves

Nucleic acids were extracted from 1 g leaf material in the presence of 5 ml of 100 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM EDTA and of 10 ml phenol/chloroform 1:1 saturated with 100 mM Tris-HCl pH 7.5. After three phenol extractions the nucleic acids were recovered by ethanol precipitation.

Gel electrophoresis, blotting, dot spotting and molecular hybridisation

Glyoxalation of the extracted nucleic acids prior to electrophoresis in polyacrylamide slab gels (5% polyacrylamide, 0.12% bisacrylamide) in 10 mM sodium phosphate, pH 6.5 as running buffer, Northern blotting, dot spotting and molecular hybridisation were performed as described by Spiesmacher *et al.* (in preparation).

Infectivity tests

Infectivity tests were carried out by mechanically inoculating the samples of cloned PSTV DNA (dissolved and diluted in a solution of 1% K_2HPO_4) onto carborundum-dusted leaves of young tomato plants in the two-leaf-stage with the aid of a glass spatula. Inoculations with the control samples and mock inoculations with buffer alone were performed in the same way. For each sample at least 10 plants were used. Immediately after the abrasive inoculation the leaves were rinsed with running tap water. For development of disease symptoms the plants were kept under semi-controlled greenhouse conditions, i.e., at temperatures between 24 and 28°C and under natural light between 30 000 and 50 000 lux. Under these conditions 2 weeks post-inoculation (p.i.) the first foliar symptoms of PSTV infection, as characterized by epinasty, malformation and veinal necrosis of leaves, appeared leading to growth retardation and stunting of the entire plant. Four weeks p.i. all plants which appeared to be healthy were decapitated (with sterile razor blades) thus inducing their dormant axillary buds to grow. In all those cases where only very low levels of infectious material are present in the inoculum, several weeks or even months may elapse before the critical concentration of viroid RNA progeny is reached in the inoculated leaves. This is required for the subsequent invasion and generalized infection of the entire plant and for the induction of disease symptoms in the newly developing leaves of the main apical shoot. Upon decapitation, however, this critical viroid concentration is reached quite rapidly in the growth-activated axillary buds because viroid replication is most active and symptom induction most pronounced in the rapidly dividing and differentiating meristematic tissue of such buds from which the axillary shoots develop.

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