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STUDIES OF POTATO SPINDLE TUBER VIROID USING AN *IN VITRO* TRANSCRIPTION SYSTEM

by

XIAONING WU

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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Date.....June 20, 1986.....

Abstract

The potato spindle tuber viroid (PSTV) causes a serious disease in higher plants such as potato and tomato. The primary sequence and predicted secondary structure of PSTV revealed that it is a small, single stranded RNA which replicates in infected plant cells. A monomeric full length cDNA copy of the PSTV genome was cloned in both of the possible orientations into the BamH1 site of the polylinker downstream from an SP6 promoter in the plasmids pSP64 and pSP65. Two types of the recombinant plasmids were constructed and named pDX1 and pDX4. After linearization of pDX1 and pDX4 at a site downstream from PSTV cDNA, *in vitro* transcription with SP6 RNA polymerase generated either (+) RNA transcripts from pDX1 or (-) RNA transcripts from pDX4. These transcripts contained the full length PSTV RNA genome plus about 20 nucleotides derived from the polylinker sequences at both ends. The reaction mixtures (plasmid DNA plus RNA transcripts) and the RNA transcripts further purified by electrophoresis on polyacrylamide gels under either denaturing or non-denaturing conditions were inoculated onto the leaves of indicator tomato plants. The mixture of (+) RNA and pDX1 DNA and the purified (+) RNA transcripts both induced the typical PSTV disease symptoms on indicator tomato plants. Since pDX1 DNA alone did not induce the disease symptoms, the infection was presumably due to the (+) RNA transcripts in both cases. Progeny viroid RNA derived from the (+) RNA transcripts was extracted from

the diseased plants. Subsequent analysis of this RNA by polyacrylamide gel electrophoresis and spot hybridization indicated that the polylinker (*i.e.* the non-viroid) sequences had been removed. The nucleic acid preparations from the diseased plants were able to induce PSTV disease symptoms on indicator tomato plants and the kinetics of the appearance of the disease symptoms was indistinguishable from those produced by inoculation of the native PSTV RNA. The observed low level of infectivity could be due to degradation of the linear RNA transcripts during the experimental procedures. The delayed appearance of the disease symptoms observed on indicator tomato plants inoculated with (+) RNA transcripts might be due to the time period required for host cells to remove non-viroid sequences from the RNA before viroid replication could be initiated. No viroid-associated disease symptoms were observed on the indicator tomato plants inoculated with (-) RNA transcripts. The lack of infectivity of the (-) monomeric PSTV RNA transcribed *in vitro* suggests that (-) monomeric PSTV RNA does not exist in the PSTV replication cycle. This result supports one of the PSTV replication models which were proposed by Branch and Robertson (1984).

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List of Abbreviations

A	Adenine
ATP	Adenosine Triphosphate
C	Cytosine
cDNA	Complementary DNA
Ci	Curie
dCTP	Deoxycytidine Triphosphate
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ehtylene Diaminetetracetic Acid
EtBr	Ethidium Bromide
G	Guanine
g	gramme
X *g*	centrifugal force
I	Inosine
l	liter
LB	Luria Broth
M	Molarity
OD	Optical Density
PSTV	Potato Spindle Tuber Viroid
RNA	Ribonucleic Acid
RNase	Ribonuclease
rNTP	Ribonucleotide Triphosphate
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulfate

T	Thymidine
tRNA	transfer Ribonucleic Acid
U	Uridine
UTP	Uridine Triphosphate
UV	Ultraviolet

1. Introduction

1.1 Viroid diseases

1.1.1 History

Martin (1922) first described the spindle tuber disease of potato at the beginning of this centaury. It is characterized by the appearance of distinct symptoms in potato plants and their tubers. The leaves of infected potato plants are somewhat darker in color and narrower than the leaves of healthy plants. They are also pointed and erect in appearance. The diseased plants grow up to only one-third to one-half of the height of normal plants. Tubers from infected plants are abnormally enlongated and smoothly skinned. They show more eyes than the normal tubers, and the eyes are sometimes located on raised knoblike areas.

The spindle tuber disease of potato does not cause total destruction of the crop or storage losses. However, it does lead to a serious reduction in total crop yield (Werner, 1926; Hunter and Rich, 1964; Singh *et al.*, 1971).

1.1.2 Discovery of potato spindle tuber viroid

Many research approaches were used in an attempt to identify the pathogen of potato spindle tuber disease. The infectious agent was present in very low concentrations, but its infectivity was extremely potent. In 1967, on the basis of its low sedimentation rate and sensitivity to

ribonuclease, Diener and Raymer (1967) reported that spindle tuber disease was caused by free RNA. In the following year, Diener (1968) showed that, *in situ*, the RNA was sensitive to digestion with ribonuclease, but not deoxyribonuclease. He concluded that the classical virion structure (i.e. nucleic acid with protein coat) for the causative agent of the spindle tuber disease was unlikely. Zaitlin and Hariharasubramanian (1970) confirmed that no identifiable coat protein was found in the disease-infected tissue. By 1972, Diener (1971, 1972) concluded from analyses of density gradients and polyacrylamide gels that the infectious agent of the spindle tuber disease was a single low molecular weight RNA species. The purified RNA was infectious and no helper virus was involved in its replication. Because of the basic differences between the potato spindle tuber disease agent and conventional viruses, Diener (1971) proposed the term "viroid" for the infectious agent of the spindle tuber disease and also for other pathogenic nucleic acids with similar properties. In 1979, Diener (1979a) gave a definition of viroid as follows;

" Viroids are low molecular weight nucleic acids that are present in certain organisms afflicted with specific maladies. Viroids are not detectable in healthy individuals of the same species but, when introduced into such individuals, they replicate autonomously and cause the appearance of the characteristic disease syndrome. Unlike viral nucleic acids, viroids are not encapsidated. Viroids

are highly resistant to heat, as well as to ultraviolet and ionizing radiation. They contain extensive regions of intramolecular complementarity and exist as covalently closed circular structures."

Therefore, the infectious RNA agent of potato spindle tuber disease was re-named as potato spindle tuber viroid (PSTV). So far, 12 different kinds of viroids have been found in nature and are listed in Table 1.

The viroid etiology of all these diseases was established by demonstrating the extra band of viroid RNA by polyacrylamide gel electrophoresis of nucleic acid preparations extracted from diseased plant tissue. In addition, the pathogenic nature of the extra RNA band was demonstrated by assaying its infectivity. The purified RNA was able to induce the corresponding disease symptoms in healthy plants (Diener, 1979a).

Table 1. The viroid diseases presently known and the abbreviations of the corresponding viroids.

[a]References: 1. Diener, 1971b; 2. Singh and Clark, 1971; 4. Semancik and Weathers, 1972a; 5. Hollings and Stone, 1973; 6. Diener and Lawson, 1973; 7. Romaine and Horst, 1975; 8. Horst and Romaine, 1975; 9. Van Dorst and Peters, 1974; 10. Sanger *et al.*, 1976; 11. Randles, 1975; 12. Randles *et al.*, 1976; 13. Sasaki and Shikata, 1977a; 14. Sasaki and Shikata, 1977b; 15. Thomas and Mohamed, 1979; 16. Dale and Allen, 1979; 17. McClean, 1931; 18. Semancik and Weathers, 1972b; 19. Galindo, Smith and Diener, 1982; 20. Chen *et al.*, 1982.

Table 1. The viroid diseases presently known and the abbreviations of the corresponding viroids

Viroid disease	Viroid	References[a]
1. Potato spindle tuber	PSTV	1,2
2. Citrus exocortis	CEV	3,4
3. Chrysanthemum stunt	CSV	5,6
4. Chrysanthemum chlorotic mottle	CCMV	7,8
5. Cucumber pale fruit	CPFA	9,10
6. Coconut cadang-cadang	CCCV	11,12
7. Hop stunt	HSV	13,14
8. Avocado sunblotch	ASBV	15,16
9. Tomato bunchy top	TBTV	17
10. Tomato apical stunt	TASV	18
11. Tomato planta macho	TPMV	19
12. Burdock stunt	BSV	20

1.2 Transmission and host range

The potato spindle tuber viroid is known to spread rapidly to neighbouring plants by a combination of wounding and contact of foliage during cultivation and handling in the field and in greenhouses (Goss, 1926a and 1926b). It has also been shown that the potato spindle tuber disease was not transmitted through the soil (Goss, 1931).

Early experiments, however, indicated that the potato spindle tuber disease could also be transmitted at low frequencies via biting insects and by aphids under field conditions (Schultz and Folsom, 1923). The results have been confirmed recently by DeBokx and Piron (1981). They showed that the two aphids species *Macrosiphum euphorbiae* and *Myzus persicae* were capable of transmitting PSTV from infected to healthy potato and tomato plants at frequencies below 6%.

Vertical transmission through seed and pollen is characteristic of viroid diseases (Singh, 1970; Hunter *et al.*, 1969). This causes serious problems in obtaining viroid-free seeds in plant breeding.

PSTV is highly infectious and is easily transmitted in experimental procedures by mechanical means using crude tissue homogenates. Tissue homogenates are usually freshly prepared with cold, slightly alkaline buffers and immediately used for inoculation. Rubbing of the inoculum onto carborundum-dusted leaves is used in inoculation procedures.

In nature, potato is the only known host exhibiting symptoms during PSTV infection. However, PSTV can infect other plant species but the hosts remain asymptomatic (Singh, 1973). The presence of viroid in other plants can be demonstrated by back inoculation of indicator plants such as tomato or potato with infected tissue extracts.

Raymer and O'Brien (1962) first discovered that the agent of potato spindle tuber disease could infect tomato plants and produce the characteristic syndrome in certain cultivars of this host. PSTV extracted from infected tomato plants was transmissible to potatoes and these potato plants developed diseased tubers. Raymer *et al.* (1964) reported that the PSTV concentration in tomato *Lycopersicon esculentum* cv. Rutgers plants was comparable to that in infected potato plants. The Rugters cultivar is therefore often used as the indicator plant for PSTV assays.

1.3 Molecular structure and composition

The early experiments based on thermodynamic and kinetic analysis as well as visualization by electron microscopy of fully denatured PSTV molecules revealed that PSTV is a single stranded covalently closed circular RNA molecule. Under non-denaturing conditions, the single circular molecule can form a highly base paired rod-like structure. The length of rod shaped native molecules is approximately half of circumference of the open circular form (McClements and Kaesberg, 1977; Sanger *et al.*, 1976).

1.3.1 The primary structure

In 1978, Gross, Sanger and coworkers (Gross *et al.*, 1978) determined the PSTV RNA sequence (359 nucleotides). Because viroids can not be sufficiently labeled with ^{32}P *in vivo* for subsequent nucleotide sequencing, the purified RNA, after controlled nuclease digestion, had to be labeled *in vitro* using γ-^{32}P-ATP and T4 polynucleotide kinase. High resolution RNA fingerprints could be obtained from complete RNase A and T1 digests of viroid RNA. It permitted the separation of these RNA fragments of PSTV and the determination of their nucleotide sequences (Gross *et al.*, 1978). Overlapping sequences were obtained by controlled digestion of PSTV RNA with a set of different RNases. The complete sequence of the 359 nucleotides of PSTV could then be established and it was clearly shown that PSTV is a single-stranded molecule.

PSTV was composed of 73 adenylyl (20.3%), 77 uridylyl (21.4%), 101 guanylyl (28.1%), and 108 cytidylyl (30.1%) ribonucleotides. No modified bases were found. From the nucleotide sequence of PSTV RNA, the molecular weight was estimated to be 115,500 (Domdey *et al.*, 1978). The AUG initiation site was missing although there were seven GUG initiation sites and six possible termination sites. There was no cap structure present. Thus, the evidence from the primary sequence suggested that PSTV RNA lacked messenger activity.

1.3.2 Secondary structure

From the primary sequence of the molecule, the most likely secondary structure of PSTV was predicted by searching for thermodynamically stable structures. A largely double-stranded, rod-like structure was obtained. The crude model was then refined by the location of cleavage sites derived from controlled enzymatic digestions of PSTV RNA. The resulting model for secondary structure consisted of a serial arrangment of 26 double-stranded stretches, which were separated by 25 segments of unpaired bases in single-stranded loops or bulge loops. There was one loop at each end of the rod-like molecule. The two partially complementary halves of the PSTV circle were virtually the same length, 179 and 180 nucleotides, respectively (Gross *et al.*, 1978).

The predicted rod-like structure was in good agreement with the electron microscopy observation (McClements and Kaesberg, 1977; Sogo *et al.*, 1973; Sanger *et al.*, 1976), the sedimentation experiments (Sanger *et al.*, 1976; Diener, 1974), and thermodynamic and kinetic studies (Sanger *et al.*, 1976; Henco *et al.*, 1977). It is now widely accepted as a model for the discussion of experimental results.

In order to search for a possible tertiary structure of PSTV RNA, dye binding studies under different ionic concentrations and specific tRNA binding to the internal loops of PSTV molecule were carried out (Riesner *et al.*, 1979; Wild *et al.*, 1980). The results from these experiments

supported the conclusion that the PSTV RNA molecule lacks a defined tertiary structure.

1.4 Mechanism of PSTV replication

1.4.1 Subcellular location

The subcellular locations of viroids in the infected host plant will give insights to their model of replication. Previous studies based on infectivity tests suggested that viroids were associated primarily with nuclear (Sanger, 1972; Takahashi and Diener, 1975) and/or membrane fractions (Semancik *et al.*, 1976). In recent years, sufficiently stringent procedures were developed for the purification of subcellular components from plant tissue (Spiesmacher *et al.*, 1983). Specific radioactive oligodexoyribonucleotide probes with sequence complementarity for distinct regions present only in either PSTV (+) or (-) RNA molecules were used to probe for the presence of PSTV RNA in various subcellular locations (Muhlbach *et al.*, 1983). PSTV (+) monomer and different oligomeric forms of PSTV (+) and (-) RNA were found in the nuclear fraction of potato cell suspension cultures. This result strongly favours the concept that the nucleus was the intracellular site of viroid replication. An average infected cell contained 200-10,000 viroid RNA molecules, depending on the progression of the disease (Schumacher *et al.*, 1983a and 1983b).

1.4.2 Messenger RNA activity

The unique structure of the PSTV molecule raises many questions related to viroid replication and function. One main question is whether viroid codes for peptides or proteins. Viroids do not carry their own replicase. Previous attempts to translate PSTV into protein in several *in vitro* protein synthesis systems has failed and viroid RNA did not interfere with the *in vitro* translation of messenger RNA species in these systems (Davies *et al.*, 1974; Semancik *et al.*, 1977). From the established primary sequence of PSTV, it appears that the circular arrangement of the uneven number of 359 nucleotides would permit three rounds of protein translation, but the common AUG initiator triplet is missing. Moreover, PSTV lacks ribosome binding sites at the 5'-side of the seven possible GUG initiator codons and does not have mRNA "cap" structures. The circularity as well as these aspects of the primary and secondary structure of PSTV argue against its translation into peptides or proteins (Gross *et al.*, 1978). The possibility that viroids might function as negative strand genomes (Matthews, 1978) has been excluded since the complementary strand of PSTV RNA does not contain AUG initiator codons. The search for viroid specific proteins in infected host tissue has also failed to reveal qualitative differences in proteins between healthy and the infected plants (Zaitlin and Hariharasubramanian, 1972).

1.4.3 Enzymes involved in viroid replication

Theoretically, viroid replication could involve transcription from either RNA or DNA templates. A DNA-mediated mechanism would require the presence of DNA sequences complementary to the entire viroid RNA and a pre-existing host RNA-dependent DNA polymerase (reverse transcriptase). Viroid replication was initially reported to be sensitive to actinomycin D in leaf tips of PSTV infected plants *in vivo* (Diener and Smith, 1975; Takahashi and Diener, 1975). On the other hand, Grill and Semancik (1980), in their studies of actinomycin D treated PSTV-infected potato tuber sprouts, concluded that the antibiotic had no specific inhibitory effect on viroid replication and that inhibitory effects previously reported were due to a general toxic effect of actinomycin D on cell metabolism. Furthermore, no reverse transcriptase activity has been detected in higher plants and viroid specific DNA sequences were not found in viroid infected hosts (Branch and Dickson, 1980; Zaitlin *et al.*, 1980). It is now generally agreed that viroids are replicated *in vivo* via oligomeric (-) RNA intermediates rather than through a DNA intermediate (Rohde and Sanger, 1981).

If viroids replicate through a complementary RNA, the RNA-synthesizing enzymes of the host should be utilized. Plant cells are known to contain two classes of these enzymes, namely the DNA-dependent RNA polymerase I, II and III, and the RNA-dependent RNA polymerase. Muhlbach and

Sanger (1979) used the selective sensitivity to α-amanitin to determine that the DNA-dependent RNA polymerase II system in the host cells was essential for viroid replication. Rackwitz *et al.* (1981) also showed that purified DNA-dependent RNA polymerase from wheat germ or from tomato leaf tissue was capable of *in vitro* transcription of viroid RNA with higher efficiency than with other natural RNA templates tested. These findings indicate that DNA-dependent RNA polymerase of the plant cell may function as RNA-dependent RNA polymerase responsible for viroid RNA synthesis *in vivo*.

Nuclear DNA-dependent RNA polymerase II, however, is not the only host enzyme capable of transcribing viroid templates. RNA-dependent RNA polymerase from healthy tomato leaf tissue accepts PSTV RNA as a template and produces full-length PSTV copies *in vitro* (Boege *et al.*, 1982). Definitive proof for involvement of these enzymes in replication of viroid RNA remains to be obtained.

1.4.4 Replication intermediates

If viroid complementary RNAs serve as templates from which progeny viroids are transcribed, they evidently must contain a full complement of the viroid sequence, that is, they must be of equal or greater length than the viroid. In fact, longer than unit length (-) RNA intermediates have been detected by nucleic acid hybridization in viroid-infected plant tissue (Rohde and Sanger, 1981; Grill

and Semancik, 1978; Owens and Cress, 1980; Branch *et al.*, 1981a). These (-) RNA strands were heterogenous in size, forming either four (Branch *et al.*, 1981a) or six (Muhlbach *et al.*, 1983) discrete bands in polyacrylamide gels. They seem to represent multimers of PSTV unit length RNA. Fingerprint analysis of hybridized RNA demonstrated that all regions of the PSTV molecules were represented in the (-) RNA strands (Zelcer *et al.*, 1982). Further characterization of the double stranded structures indicated that monomeric circular and linear (+) PSTV strands were complexed with multimeric (-) RNA strands and the synthesis of the double-stranded complexes increased stimultaneously with the synthesis of the single-stranded PSTV RNA (Owens and Diener, 1982). Also, as soon as (+) strands of PSTV RNA could be detected, multimeric (-) strands were also present (Branch and Robertson, 1984).

These results support the hypothesis proposed by Branch *et al.* (1981a), that the longer than unit length (-) RNA strands play a role in viroid replication. The (-) RNA strand in the double stranded (+) and (-) RNA complexes represented replication intermediates. The (-) RNA strands consists of tandem repeats of approximate unit length viroid RNA.

If the multimeric (-) RNA strands of PSTV are involved in viroid replication, it is possible that these strands would give rise to (+) strands with equal or larger size (Branch, 1981b). Recent studies have shown that monomer,

dimer, and trimer length PSTV-specific (+) RNAs were all present, and may serve as precursors of mature circular progeny RNA (Branch and Robertson, 1984).

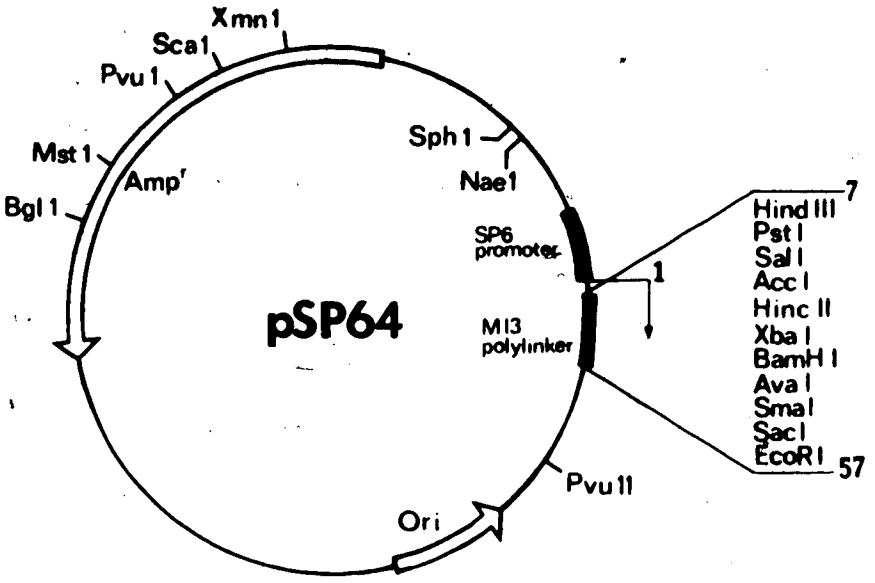
Cleavage and ligation of multimeric (+) RNA plays a direct role in viroid replication. Konarska *et al.*(1981) first showed that RNA ligase isolated from wheat germ was able to circularize linear RNA by the formation of a 2'-phosphomonoester, 3', 5'-phosphodiester linkage. This wheat germ RNA ligase requires a 2', 3' cyclic phosphodiester substrate for highly efficient ligation to a free 5' phosphorylated end. The RNA ligase was able to efficiently circularize PSTV linear RNA *in vitro* (Branch *et al.*, 1982; Kikuchi *et al.*, 1982). It is possible that the enzymes similar to wheat germ RNA ligase may exist in plant cells and could circularize linear PSTV RNA.

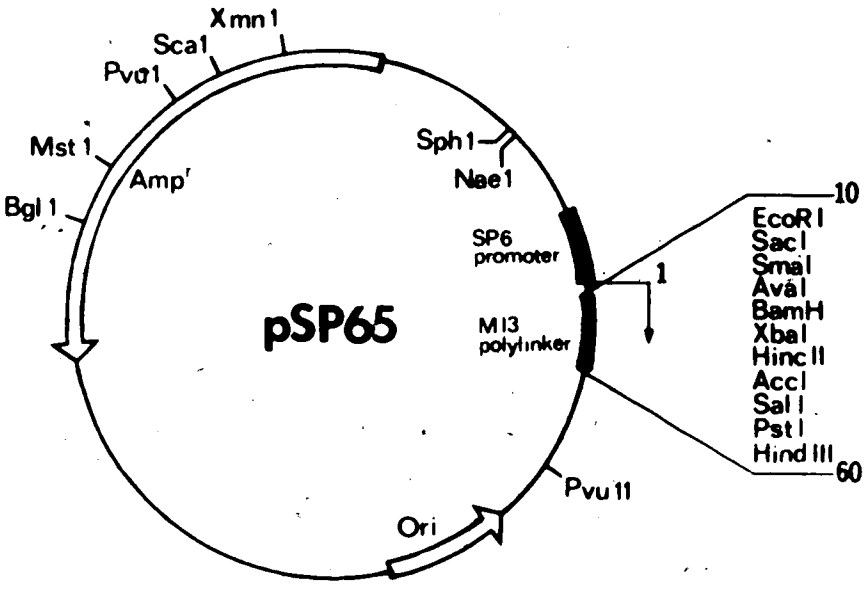
1.4.5 Studies of the PSTV RNAs by using the SP6 transcription system

Recently, the SP6 system has been used for the efficient and specific transcription *in vitro* of cloned DNA into single-stranded RNA. This has made it possible to study viroid RNAs and their replication intermediates. The DNA-dependent RNA polymerase of bacteriophage *Salmonella typhimurium* 6 has been purified (Bulter and Chamberlin, 1982) and found to specifically recognize its own phage promoter for transcription (Kassavetis *et al.*, 1982). Vectors have been constructed containing the SP6 promoter

followed by the M13 polylinker sequence cloned in either orientation (pSP64 and pSP65) (Melton *et al.*, 1984). Thus, any DNA fragment can be inserted into the polylinker. After appropriate linearization of the DNA fragment by digestion with restriction endonucleases, a large amount of single-stranded RNA can be syntnesized by *in vitro* "run-off" transcription using SP6 RNA polymerase. Fig. 1 shows the physical maps of pSP64 and pSP65 plasmids.

Fig. 1. Restriction endonuclease maps for SP6 cloning vectors pSP6 and pSP65. The numbers (7 and 57, 10 and 60) indicate the number of nucleotides from the transcriptional startpoint.


Xmn1
Sca1
Pvu1
Mst1
Amp'
Bgl1
Sph1
Nae1
SP6 promoter
MI3 polylinker
pSP64
Ori
Pvu11
1
7
Hind III
Pst I
Sal I
Acc I
Hinc II
Xba I
BamH I
Ava I
Sma I
Sac I
EcoR I
57


Xmn1
Sca1
Pvu1
Mst1
Amp'
Bgl1
Sph1
Nae1
SP6 promoter
MI3 polylinker
pSP65
Ori
Pvu11
1
10
EcoR I
Sac I
Sma I
Ava I
BamH
Xba I
Hinc II
Acc I
Sal I
Pst I
Hind III
60

Robertson *et al.*(1985) first reported on the studies of viroid RNA processing and generation of viroid progeny RNA molecules by the SP6 *in vitro* transcription system. A dimeric cDNA of PSTV was cloned into a plasmid pSP65. *In vitro* transcription was carried out and produced a single linear RNA, which includes two copies of PSTV RNA with about 20 nucleotides of vector sequence at each end. When the ^{32}P-labelled RNA was incubated in a neutral Tris-HCl buffer containing ammonium and magnesium ions, a self-catalyzed cleavage occurred to produce unit-length linear PSTV RNA and two small pieces of RNA. When incubated with wheat germ RNA ligase, the unit-length cleavage product gave a high yield of circular molecules. The RNA fingerprint data showed that the circular molecules were indistinguishable from PSTV circules arising *in vivo*. In addition, circularization by the wheat germ ligase suggested that a 2', 3' cyclic phosphate group was generated during the cleavage reaction of the transcript. These results and previous studies suggest that the processing of a multimeric precursor into a mature circular viroid can be completed by autocatalytic cleavage followed by ligation (Robertson *et al.*, 1985).

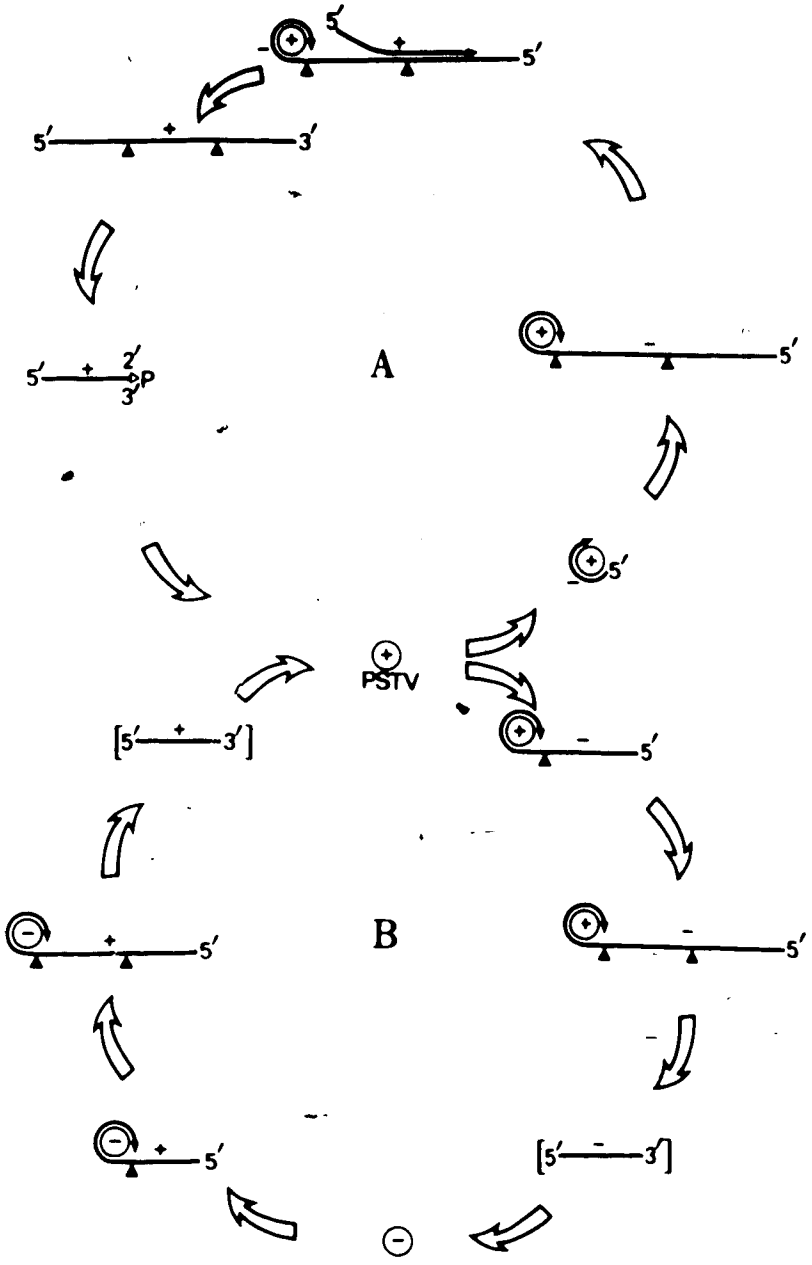
Tabler and Sänger (1985) used the same *in vitro* transcription system to study replication intermediates of PSTV RNA. PSTV multimeric (+) and (-) RNAs were transcribed *in vitro* by SP6 RNA polymerase. Infectivity tests have revealed that the multimeric (+) RNA had the same infectivity as natural PSTV RNA and that unit-length PSTV

RNA was regenerated *in vivo*. On the other hand, the corresponding multimeric (-) RNA did not initiate PSTV replication in the host cell. From these results, they concluded that PSTV multimeric (+) RNA served as the precursors of monomeric circular progeny RNA. Apparently cleavage by either autocatalytic mechanism or by pre-existing host enzymes followed by ligation of RNA are required. However, the non-infectious multimeric (-) RNA might not be cleaved and circularized *in vivo*. Presumably it served only as an intermediate template for PSTV (+) RNA synthesis.

1.4.6 Replication model

A number of mechanisms have been proposed to explain the replication of viroids. Brown and Martin (1965) proposed a model with circular RNA as a replication template for animal RNA viruses. This "rolling circle" model was later applied to replication of circular single-stranded DNA molecules such as bacteriophage ΦX174 (Gilbert and Dressler, 1968). From the evid[illegible] multimeric PSTV (-) strands as well as other rece[illegible] Branch and Robertson (1984) developed a hypoth[illegible]cation cycle to describe the replication of PSTV [illegible] viroids (Fig. 2).

Fig. 2. A model for the replication of PSTV RNA proposed by Branch and Robertson (1984). [ ] indicates putative unstable reaction intermediates.


5′
5′
5′
3′
5′
2′
P
3′
A
5′
5′
PSTV
[5′ 3′]
5′
B
5′
5′
[5′ 3′]
5′

Scheme A: the process of PSTV RNA replication steps are summarized as follows.

(1) an infectious circular (+) strand enters into the cell.

(2) a multimeric complementary (-) strand is synthesized from the (+) strand.

(3) the multimeric (-) strands serve as the template for the production of multimeric (+) strands.

(4) the multimeric (+) strands are cleaved to produce unit length molecules.

(5) the unit length molecules are circularized to give progeny circles.

Scheme B: the major difference with scheme A is that circular (-) and unit-length (-) RNA would exist in the infected cells. In Northern blot experiments, unit length (-) strands could be detected, but they did not form a prominent band (Branch and Robertson, 1984). The existence of circular (-) RNA has not been reported yet. The authors suggested that the unit-length (-) RNA is an unstable reaction intermediate.

At present, these schemes for viroid replication could explain the viroid-specific nucleic acid species detected in PSTV-infected plants. The following features are the molecular mechanisms known about the viroid RNA replication (Branch and Robertson, 1984; Cress *et al.*, 1983):

(1) PSTV and other viroids replicate through direct RNA to RNA transcription.

(2) Both multimeric (+) and (-) strands are produced during viroid infection. This implies that a rolling circle mechanism is used for the synthesis of at least one of these strands.

(3) These (+) and (-) strands and progeny (+) strand viroids, are synthesized by pre-existing host enzymes, possibly by the DNA-dependent RNA polymerase II functioning as an RNA-dependent RNA polymerase.

(4) Specific cleavage and ligation may be required to generate progeny monomeric circular RNA molecules from the multimeric (+) strands.

1.5 Mechanism of viroid pathogenesis

The small RNA genomes of viroids are not capable of protein synthesis due to the absence of the initiation triplet AUG from both the (+) and (-) strand sequences. Moreover, PSTV has failed to stimulate nor inhibit protein synthesis *in vitro* and no viroid-specific protein has so far been detected *in vivo*. The nuclear location of viroids suggests that their pathogenicity in higher plants may be due to direct interaction of the viroid RNA with the host genome. However, the mechanism of viroid pathogenicity is still unknown.

1.5.1 Infectivity of PSTV

It is known that the viroids have unsually high specific infectivity. Diener and Raymer (1967) demonstrated

infectivity in partially purified PSTV preparations containing as little as 5 X 10^{-4} μg of total nucleic acid per ml. Inoculation of plants with purified viroids further confirmed the extremely high specific infectivity of these pathogens. Sanger *et al.*(1976) found that inoculation of tomato plants with 50 to 100 RNA molecules per plant resulted in 10% of the plants becoming infected. Wounding of cells is essential for viroid infection to occur.

Electron microscopy of denatured PSTV and electrophoresis of formamide-denatured PSTV in polyacrylamide gels equilibrated with 50% formamide and 6M urea (Owens *et al.*, 1977) revealed two types of molecules in PSTV RNA preparations: a covalently closed circular form and a linear form. Bioassay of individual slices taken from the region of the gels containing the two PSTV bands showed that the linear molecules of PSTV were also infectious. If the linear molecules were derived from circular ones and act as the intermediates in viroid replication (Branch and Robertson, 1984), it is expected that they would be infectious and it may suggest that nicking at a specific site is a natural maturation step. However, as Sanger suggested (Sanger *et al.*, 1979), the linear molecules may be generated by nicking of circles during isolation procedures. If so, it is possible that randomly nicked molecules might be reformed into circular forms by a host RNA ligase *in vivo*. The existence of covalently linked circular RNA molecules *in vivo* suggests that an RNA ligase exists in the

host plants of viroids (Diener, 1979a).

Recently, a monomer and dimer cDNA of PSTV cleaved at HaeIII restriction endonuclease site were cloned into a plasmid adjacent to the bacterial Lac promoter (Cress *et al.*, 1983). The PSTV RNA was transcribed in *E. coli* and the total RNAs were extracted and inoculated onto tomato plants. Bioassay and hybridization experiments have shown that the monomer and dimer (+) RNAs of PSTV transcribed in *E. coli* were infectious. However, the infectivity of the (+) monomer RNA was very low and (-) monomer as well as (-) dimer RNA of PSTV extracted from *E. coli* appeared to be non-infectious (Cress *et al.*, 1983). It is interesting to note that the dimeric and multimeric (+) RNAs of PSTV transcribed *in vitro* have been unequivocally demonstrated to be infectious and produced unit-length viroid RNA *in vivo*, whereas the corresponding multimer (-) RNA was non-infectious (Robertson *et al.*, 1985; Tabler and Sanger, 1985). The significance of a low level of infectivity for monomer viroid (+) RNA and the mechanism for generating unit-length infectious viroid RNA *in vivo* still await further analysis.

Cress *et al.* (1983) demonstrated that a dimeric cDNA of PSTV was infectious. One year later, Sanger and his colleagues cloned the cDNA of PSTV into the bacteriophage M13mp8 and M13mp11 vectors as monomers, dimers and random multimers. Their experiments not only confirmed Cress' findings, but also provided the following surprising results (Tabler and Sanger, 1984): (1) it was found that not only

double-stranded but all M13 cloned dimeric and multimeric single-stranded PSTV DNAs were infectious irrespective of whether they represented the PSTV (+) or (-) DNA strand. (2) the cloned single as well as double-stranded monomeric PSTV cDNAs were infectious in one orientation but not the opposite orientation. (3) non-infectious subgenomic fragments of cloned PSTV cDNA were capable of initiating infection when inoculated as a mixture. Fractionation of the nucleic acids extracted from the inoculated plants on polyacrylmide gel and dot-spot hybridization with alkaline-treatment indicated that the input PSTV cDNA did not replicate. However, viroid RNA was apparently regenerated and predominantly circular and some linear PSTV RNA molecules of unit-length accumulated (Tabler and Sanger, 1984). Undoubtedly, infectious viroid-specific cDNA will become a versatile tool for studying viroid replication and pathogenesis.

1.5.2 Correlation between viroid pathogenicity and structure

Different PSTV field isolates exist which produce disease in tomato plants characterized by either very mild, mild, intermediate or severe symptoms and by the death of the infected plants. The severe strain causes general stunting of potato plant growth, deformity of the upper foliage, and production of disfigured potatoes. Mild strains of PSTV produce barely detectable symptoms (Fernow, 1967). Nucleotide sequence analysis of a mild strain of PSTV

revealed that its sequence differs from that of the severe strain by only three nucleotides (Gross *et al.*, 1981). In positions 120 and 121, AA was replaced by U; in position 310, A was replaced by U; and between position 312 and 313, a U was inserted. Thus, the total number of nucleotides was identical in the two strains. No marked change in the secondary structure between the mild and severe strains was observed (Gross *et al.*, 1981). The obvious difference in pathogenicity between the two strains, therefore, seems to be due to the nucleotide changes rather than changes in the secondary structure of the molecule.

Rohde *et al.*(1981) used reverse transcriptase to synthesize full length PSTV for analysis of different PSTV isolates at the DNA level by the Maxam-Gilbert DNA sequencing technique. The comparison of the established nucleotide sequences of various PSTV isolates of different virulence showed that in all isolates the total number of 359 nucleotides was strictly conserved. Nucleotide exchanges, insertions and deletions, however, were found in these isolates when using the strain producing the mildest symptoms as a standard. All changes were observed in only three regions of the PSTV primary sequence *i.e.*, at nucleotides 40-55, 115-125 and 305-320. In the predicted secondary structure, these regions were located in two distinct domains, one on the left and the other one on the right hand side of the rod-shaped molecule (Rohde *et al.*, 1981; Sanger, 1984). The number of nucleotide changes in the

left hand side of the various isolates was found to increase with increasing pathogenicity. These nucleotide changes resulted in a decrease of the number of base pairs, leading to increasing structural instability. The change observed in the right hand side of the PSTV structure always involved the same uridine which was replaced by two adenosines. Evidently, these changes in the nucleotide sequences are directly correlated with changes in pathogenicity.

1.5.3 Possible model of viroid pathogenesis

Semancik and Weathers (1972) first speculated that viroids might be some "regulatory" RNA that has "escaped" and acquired the ability of self-replication. This hypothesis was supported by the comparison of the established viroid sequences with the sequence of the small stable nuclear U1a RNA (Diener *et al.*, 1982; Gross *et al.*, 1982). U1a RNA is 165 nucleotides long (Reddy *et al.*, 1974) and is strictly conserved in its primary sequence during evolution (Bralant *et al.*, 1980). It is assumed that U1a RNA plays a key role in the splicing of eukaryotic mRNA by complementarity of its 5' end sequence to the consensus sequence at the 5'- proximal region of the intron sequences of mammalian genes so that the two coding regions of the mRNA precursor are brought together to allow that splicing to occur (Lerner *et al.*, 1980; Rogers and Wall, 1980).

Striking similarity occurs between ACCUG sequences of U1a RNA and ACCCG sequences in several locations on all

viroids, with the exception of Avocado sunblotch viroid (Gross *et al.*, 1982). This suggests that viroids could bind to these recognition sites on the pre-mRNA that are recognized by U1a RNA and thus interfere with mRNA splicing. Based on these considerations, Sanger (1984) proposed the viroid interference with mRNA synthesis and processing as a molecular basis of pathogenicity. This hypothesis was supported by the *in vitro* transcription experiments (Rackwitz *et al.*, 1981). In those experiments, viroid RNA was transcribed *in vitro* with higher efficiency by DNA-dependent RNA polymerase II from tomato and wheatgerm than other natural RNA templates tested. In the hypothetic model it was proposed that (+) and (-) strand viroid RNA molecules can act as competitors for DNA-dependent RNA polymerase II during RNA synthesis and therefore prevent mRNA synthesis from the host DNA templates. Moreover, the viroid RNAs can compete directly with U1a RNA for mRNA precursors to block normal mRNA splicing process (Sanger, 1984).

It should be mentioned that any model of viroid pathogenesis must not only account for the observed pathological consequences of viroid infection, but also account for the fact that in certain plant species, viroids are replicated efficiently without any expression of disease symptoms (Diener, 1983). Furthermore, the model should consider the phenomenon of cross-protection in viroid pathogenesis, that is, the host plants infected by mild

strains are somehow protected from developing symptoms following subsequent inoculation with severe strains (Niblett *et al.*, 1978).

Pathogenicity of viroids is a complex biological phenomenon and the expression of disease symptoms depends on the interaction between the viroid and host cell. For instance, different tomato cultivars may respond with severe (cv. Rutgers), mild (cv. Rentita), or practically no symptoms (cv. Hilda 72) upon infection with the same PSTV isolate, although RNA replication and accumulation are at about the same level in all three cultivars (Sanger, 1984). Moreover, symptom development is dependent on a threshold concentration of viroid RNA and also on enviromental conditions. High temperature, high light intensities, and inoculation with highly infectious inoculum generally increase the severity of symptoms and rapidity of their appearance (Sanger and Ramm, 1975; Sanger, 1980).

1.6 Diagnosis and control of viroid disease

In the past, the only available method for diagnosis of viroid diseases was bioassay on sensitive indicator hosts, for example tomato (cv. Rutgers) for the detection of PSTV. These assays are slow and unreliable (Fernow, 1967). Polyacrylamide gel analysis has been used for diagnosis by detection of viroid RNA in extracts of crop plants (Morris and Wright, 1975; Morris and Smith, 1977; Pfannenstiel *et al.*, 1980; MacQuair *et al.*, 1981). The silver-staining

method (Sammons *et al.*, 1981) has improved the detection sensitivity to 1 ng per band of nucleic acids in polyacrylamide gels (Sommerville and Wang, 1981; Goldmann and Merril, 1982). However, gel electrophoresis is laborious, expensive and therefore not suitable for the rapid screening of large numbers of viroid affected plants.

Nucleic acid hybridization for the detection of PSTV in potato tubers has been developed using radioactive PSTV-specific cDNA as a hybridization probe (Owens and Cress, 1980). No purification of nucleic acids was required. As little as 5 µl of clarified sap from plant tissue spotted onto a nitrocellulose membrane was sufficient. The hybridization method was about 10 times more sensitive than detection of a stained viroid RNA band by polyacrylamide gel electrophoresis (Owens and Diener, 1984). This technique was applicable for the detection of PSTV or other viroids in large number of samples.

In practice, the control of viroid diseases is mainly based on prevention rather than cure. The preventive measures include growing of crops from viroid-free seeds or stocks and preventing viroids from entering and spreading through crops (Gibbs and Harrison, 1976). Since high temperatures enhance viroid replication, Lizarraga *et al.* (1980) have shown that PSTV can be eliminated from potato tubers by prolonged storage (1-3 months) of the tubers at 5°C, followed by apical meristem culture. This "cold-treatment" method resulted in about half of the

regenerated plants being viroid-free.

1.7 Possible origin of viroids

Initially, it was speculated that viroids originated from conventional viruses by degeneration, or alternatively represented very primitive viruses (Diener, 1974, 1979b). These possibilities became less likely after the unique primary and secondary structures of viroids were established. Another hypothesis (Hadidi *et al.*, 1976) suggested that PSTV originated from host genetic material. This is no longer tenable because viroid-specific DNA could not be found in either healthy or viroid-infected plants (Branch and Dickson, 1980; Zaitlin *et al.*, 1980; Rohde and Sanger, 1981). Two major hypotheses on viroid origin can be discussed in light of recent knowledge.

Diener (1974, 1979a) suggested that viroids might have originated from low molecular weight nuclear RNAs. These RNAs perform normal regulatory functions within the cell nucleus. Mutations of these RNAs to allow their replication and/or introduction into a foreign plant species because of human agricultural and horticultural activities could have changed these normal RNAs into pathogenic ones.

Several authors (Diener, 1979a, 1981; Gross *et al.*, 1982; Roberts, 1978; Crick, 1979) proposed that viroids may have originated during RNA splicing processes from spliced out and circularized introns. These introns were somehow able to both escape degradation and become efficiently

replicated in the host cell. This "escaped introns" hypothesis is supported from the finding of surprisingly similar sequence homology between the small nuclear U1a RNA and PSTV RNA. However, it should be pointed that none of the U1a RNA sequences are present in the plus or minus strand of ASBV (Symons, 1981). Therefore, this viroid may be of different origin. The hypothesis also does not explain the fact that a viroid may be pathogenic in some hosts but harmless in others. Furthermore, there is no direct evidence as yet that U1a RNA exists in plants.

Besides these hypotheses, several other hypotheses about the origin of viroids have been discussed. For example, Sanger (1984) proposed that viroids might have evolved as RNA pathogens through the infection of higher plants by prokaryotes. Zimmern (1982) speculated that viroids might come from a novel class of RNA elements that exchange genetic information between eukaryotic cells. It should be stressed that these hypotheses are theoretical and speculative. Questions about the origin of viroids will wait to be answered until we have more understanding not only of viroids themselves, but also of control and regulation in eukaryotic cells.

1.8 The rationale of this study

Despite the wealth of information on the molecular and structural features of PSTV, little is known about its mechanisms of replication and pathogenesis or the

interaction between PSTV RNA and components of the host cell. Branch and Robertson (1984) proposed two alternative models (schemes A and B) for PSTV RNA replication, but several steps in the replication cycle remain unclear, particularly in scheme B. Nucleic acid hybridization experiments have clearly demonstrated the existence of (+) and (-) multimeric RNAs of PSTV replicative intermediates in PSTV infected plant cells (Branch *et al.*, 1981a; Muhlbach *et al.*, 1983; Branch and Robertson, 1984). The (+) multimeric PSTV RNAs transcribed *in vitro* are infectious and produce progeny viroid RNA when inoculated onto tomato plants (Robertson *et al.*, 1985; Tabler and Sanger, 1985). These results strongly suggest that the circular (+) form of PSTV RNA is generated from the (+) multimeric RNAs of replicative intermediates. However, the two alternative models can not be distinguished solely on the basis of these results. The key to distinguishing between these models would be to determine whether or not the (-) multimeric RNA of PSTV replicative intermediate can produce the (-) monomeric RNA during the PSTV replication cycle.

Two approaches have been used to attempt to solve this problem. One of them was to test the infectivity of (-) multimeric PSTV RNA. Tabler and Sanger (1985) produced the (-) multimeric RNAs of PSTV by using the SP6 *in vitro* transcription system and inoculated them onto indicator tomato plants. Unlike the (+) multimeric RNAs of PSTV, these (-) multimeric RNAs of PSTV failed to initiate normal PSTV

replication. No disease symptoms were observed and no (+) and (-) PSTV RNAs were detected by Northern blot analysis in these plants inoculated with these (-) multimeric PSTV RNAs. This result suggests that the (-) multimeric RNAs of PSTV only serve as replication intermediates, and can not produce the (-) monomeric RNA of PSTV. Obviously, this finding favors scheme A of the above mentioned PSTV replication models. Another approach would be to demonstrate the infectivity of (-) monomeric PSTV RNA. Cress *et al.* (1983) first reported that the (-) monomeric PSTV RNA transcribed in *E. coli* is non-infectious. In Cress' experiments, however, the precise size of the transcribed PSTV RNAs was not defined and these RNAs were not "run-off" transcripts. Therefore, the transcripts might have carried a large segment of the plasmid sequence that could have affected viroid replication. Moreover, the RNA transcripts were not purified. Total RNAs extracted from *E. coli* were inoculated directly onto indicator tomato plants.

In this study, to further study the (+) and (-) monomeric PSTV RNAs transcribed *in vitro*, the cDNA of PSTV was cloned into SP6 vectors and transcribed in an *in vitro* system. The (+) and (-) monomeric PSTV RNA transcripts were obtained *in vitro* by using SP6 RNA polymerase. These RNA transcripts were characterized, purified on polyacrylamide gels and used for infectivity tests on indicator tomato plants. The infectivity of these RNA transcripts was examined by the development of the PSTV disease symptoms and

confirmed by detection of PSTV RNA with hybridization and gel electrophoresis methods. The demonstration of the infectivity of the PSTV monomeric RNAs transcribed *in vitro* can help in understanding the mechanism of viroid replication. In addition, the ability to generate these infectious monomeric PSTV RNAs is potentially useful for studying the relationships between viroid structures and functions.

2. Materials and methods

2.1 PSTV cDNA clone and viroid strains

The PSTV cDNA clone, pAV401, used in this study was kindly provided by Drs. D. K. Lakshman and C. Hiruki, Department of Plant Science, University of Alberta, Edmonton, Canada. This clone was originally obtained from Dr. Pieter Vos of the Agricultural University, Wageningen, Netherland. It contained the full-length (359 base pairs) cDNA of the Holland strain of PSTV cloned into the BamH1 restriction endonuclease site of plasmid vector pBR322 (Van Wezenbeck *et al.*, 1982).

The severe strain of PSTV used in this study was isolated from diseased potato plants in Alberta by Dr. C. Hiruki. This PSTV strain when inoculated on indicator tomato plants (*Lycopersicon esculentum* cv. Rutgers) resulted in the typical viroid disease.

Digestion of DNA with restriction endonuclease enzymes obtained from Bethesda Research Labs (BRL, distributed by Gibco Canada Ltd., Ontario) and Pharmacia (Pharmacia Canada Ltd., Dorval, Quebec) was carried out according to the instructions of the manufacturers.

2.2 Purification of plasmid DNA

The following protocol (Clewell and Helinski, 1972; Maniatis *et al.*, 1982) was used for the purification of pAV401 and recombinant pSP6 vectors containing PSTV

monomeric cDNA in two opposite orientations. The purified plasmids were sufficiently pure for transcription *in vitro*.

E. coli strain HB101 transformed with the plasmid DNA was cultured for 12-16 hours at 37°C in Luria broth (LB) medium (Bacto-tryptone, 10 g; Bacto-yeast extract, 5 g; NaCl, 5 g; adjusted to a final volume of one liter with water) with 40 μg/ml ampicillin. Ten milliliters of the culture was transferred to 500 ml M9 medium (Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; 2 ml of 1M $MgSO_4$, 10 ml of 20% glucose, 0.1 ml of 1M $CaCl_2$, pH 7.4, adjusted a final volume of one liter with water) containing 40 μg/ml ampicillin and grown at 37°C with vigorous shaking until the culture reached an OD_{600} of about 0.4. Plasmid amplification was performed by adding 50 μg/ml chloramphenicol to the culture with vigorous shaking for a further 14-16 hours. The cells were harvested by centrifugation at 4,000 X *g* for 10 minutes at 4°C in 250 ml polycarbonate bottles and washed with 1 X TNE buffer (0.01M Tris-HCl, pH8.0, 0.1M NaCl and 1mM EDTA), then centrifuged again as before. The cell pellet derived from 200 ml of bacterial culture was resuspended with 3 ml of mix K (25% sucrose, 0.05M Tris-HCl, pH8.0) and transferred into 10 ml polycarbonate tubes. One ml of 0.2M EDTA, pH8.0, and 0.2 ml of 10 mg/ml egg white lysozyme (Sigma), freshly prepared in 0.25M Tris-HCl, pH8.0, was added. The solution was incubated at room temperature for 30 minutes. Then 4 ml of lysis buffer (0.05M Tris-HCl, pH8.0, 0.05mM EDTA and 0.2% Triton X-100) was added to the

solution. The mixture was rotated at room temperature until complete lysis occurs. High molecular weight DNA and bacterial cell debris were removed by centrifugation in the Ti80 rotor of a Beckman L8-80 ultracentrifuge at 25,000 rpm (45,000 X *g*) for an hour at 4°C. The supernatant was transferred to a 15 ml plastic graduated centrifuge tube and the volume was adjusted to 8 ml with lysis buffer. Solid cesium chloride (7.85g) was added to adjust the density of the solution to about 1.55 g/ml. The solution was transferred to a 10 ml Beckman quick seal centrifuge tube and mixed with 0.67 ml of 3 mg/ml ethidium bromide (EtBr) solution. The remainder of the tube was filled with light paraffin oil. Centrifugation was performed in the Ti80 rotor of a Beckman L8-80 ultracentrifuge at 35,000 rpm (90,000 X *g*) for 48 hours at 20°C. The plasmid DNA band was visualized by UV illumination (Spectroline, TR-302) and collected into Epperdorf centrifuge tubes. The collected solution was extracted with an equal volume of N-butanol until all the pink color disappeared from the aqueous solution. It was then dialysed against 1 l of TE buffer (0.01M Tris-HCl, pH7.5, 0.001M EDTA) at 4°C with 3 changes of buffer. The DNA solution was recovered from the dialysis bag and extracted with an equal volume of phenol saturated with TNE, followed by extraction with ether. A tenth volume of 3M NaOAc, pH8.0, and 2.5 volumes of 95% ethanol was added to the DNA solution for precipitation at -20°C overnight. The pellet was collected by centrifugation at 12,100 X *g* for 10 minutes at

4°C, and washed with 70% ethanol containing 0.125M NaOAc, pH8.0. The DNA pellet was transferred, collected by centrifugation in an Eppendorf centrifuge and dried under vacuum. Finally, the DNA pellet was resuspended in an appropriate volume of TE buffer and the values of OD_{260} and OD_{280} were measured. The DNA was then ready for further analysis of digestion by restriction endonucleases.

2.3 Purification of the full length DNA copy of PSTV

PSTV cDNA was obtained by digestion of pAV401 with restriction endonuclease BamH1 (Pharmacia). Separation and recovery of the cDNA copy was carried out by gel electrophoresis in 1.5% low melting point agarose (BRL), followed by extraction of the BamH1 DNA fragment (Maniatis *et al.*, 1982). One µl of 10 mg/ml yeast tRNA was added as carrier in ethanol precipitation of the DNA fragment. The PSTV cDNA served as either a fragment for subcloning into a pSP64 plasmid or a probe for nick translation for use in colony and dot-blot hybridization.

2.4 Subcloning the full length cDNA copy of PSTV into pSP6 plasmids in opposite orientations

) Subcloning cDNA copy of PSTV into pSP64 and pSP65 vectors

The pSP64 plasmid DNA was digested overnight with BamH1 enzyme (Pharmacia) and the DNA fragments were purified by gel electrophoresis in 0.7% low melting point agarose

(Maniatis *et al.*, 1982). The linearized pSP64 plasmid DNA was dephosphorylated by incubating with bacterial alkaline phosphatase (BRL) at 65°C for 10 minutes followed by phenol extraction and ethanol precipitation (Maniatis *et al.*, 1982). The dephosphorylated plasmid was incubated with the PSTV cDNA in the presence of T4 DNA ligase (Pharmacia, about 0.5 Weiss unit per microgram DNA) and ligation buffer (0.5 mM Tris-HCl, pH7.6, 10mM $MgCl_2$, 10mM dithiothreitol and 1mM ATP) at 4°C for 12-16 hours. Bacterial *E. coli* strain HB101 was used for transformation of the ligated plasmid. After transformation, the bacteria were plated on LB medium containing 0.8% agar and 25 μg/ml ampicillin (Maniatis *et al.*, 1982). The recombinant clones which had PSTV cDNA inserts were identified by colony hybridization. SmaI restriction endonuclease (BRL) was used to determine the orientation of PSTV cDNA inserts in the pSP64 plasmid (see explainations in detail in Section 3, Chapter 3).

In order to obtain the recombinant clones which contain PSTV cDNA in opposite orientation, the recombinant pSP64 plasmid was first digested with HindIII enzyme (Pharmacia) overnight. Completeness of digestion was checked by gel electrophoresis in 0.7% agarose. The linearized plasmid was recovered by ethanol precipitation and further digested with EcoRI enzyme (Pharmacia) for another 12 hours. Then, the small HindIII and EcoRI DNA fragment was purified by gel electrophoresis in 1.5% low melting point agarose and recovered by heating and phenol extraction followed by

ethanol precipitation. This small DNA fragment was incubated with the pSP65 plasmid digested with HindIII and EcoRI endonucleases in the presence of T4 DNA ligase (Pharmacia) as described before. *E. coli* strain HB101 was also used as a competent cell for transformation. Screening of the recombinant clones was performed by dot-blot hybridization. The cloning principle was described in Section 3, Chapter 3.

(2) Preparation of the nick translation probe

The cDNA fragment of PSTV with BamH1 sticky ends was obtained from pAV401 plasmid as described above. The nick translation kit was supplied by Amersham Canada Limited (Cat. No. N.5000). Ten μl of enzyme solution, 10 μl of nucleotide solution, 2 μl of probe DNA (about 0.5 μg), 48 μl of sterilized water and 20 μl of α-^{32}P dCTP (Amersham Canada Limited, Cat. No. PB10205, 10 mCi/ml, about 3000 Ci/mmol) were mixed together and the reaction was incubated at 14°C for 4 hours. Twenty μg of yeast tRNA, 10 μl of 3M NaOAc and 340 μl of 95% ethanol were added to the solution after incubation. The mixture was kept at -70°C for 1 hour, and then centrifuged for 15 minutes. The pellet was dried under vacuum, and then dissolved in 100 μl of TE buffer. The ^{32}P-labeled probe was then ready for hybridization. Before use, an equal volume of deionized formamide was added and the probe was denatured in a boiling water bath for 5 minutes.

(3) Colony hybridization

In order to identify the bacterial colonies which contained PSTV cDNA inserts, colony hybridization was used for screening according to Maniatis *et al.* (1982) with slight modifications.

The bacterial colonies were randomly picked and transferred onto LB plates containing 0.8% agar and 25 µg/ml ampicillin and grown overnight at 37°C. A Biodyne transfer membrane (Pall Ultrafine Filtration Corportion, P/N BNNG2225) was placed on top of the plate. The colonies were transferred to the membrane by pressing the membrane gently. If the transferred colonies were less than 2 mm in diameter, the membrane was left to incubate at 37°C for 1-2 hours.

Four layers of Whatman 3 MM paper were placed inside 100 mm Petri dishes and saturated with 10% SDS. Three other Petri dishes contained (a) denaturing solution (0.5M NaOH, 1.5M NaCl), (b) neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, pH8.0) and (c) 2 X SSPE (1 X SSPE is 0.18M NaCl, 10M sodium phosphate, pH7.4, 1mM EDTA). The membrane was laid upon the 10% SDS for 5 minutes, and then left for 10 minutes in the denaturing solution, another 10 minutes in the neutralizing solution and finally 10 minutes in the 2 X SSPE solution. Then the membrane was placed onto dry Whatman 3 MM filter paper, left for 1 hour at room temperature and baked in an oven at 80°C for 2 hours. The dry membrane was floated on the surface of a tray containing 6 X SSC (1 X SSC is 0.15M NaCl, 0.015M sodium citrate, pH7.0) until wetted from beneath. Five minutes later, the membrane was

transferred into a small rectangular container containing 100 ml of prewashing solution (1M NaCl, 1mM EDTA, 0.1% SDS, 50mM Tris-HCl, pH8.0) and incubated at 42°C. After 30 minutes of incubation, the membrane was placed (colony side up) on a glass plate and scraped gently but forcefully with a ruler to remove cell debris from the surface of the membrane. Finally, the membrane was returned to the prewashing bath and incubated at 42°C for another 2 hours.

After incubation, the membrane was transferred into a plastic bag containing 5 ml of prehybridization buffer [2.5 ml of 100% deionized formamide, 1.75 ml of 20 X SSPE, 0.5 ml of 50 X Denhardt's solution (Ficoll, 5 g; polyvinylpyrrolidone, 5 g; bovine serum albumin, 5 g; adjusted a final volume of 500 ml with water), 0.05 ml of 10 mg/ml denatured salmon sperm DNA (Sigma) prepared according to Maniatis *et al.* (1982), 0.05 ml of 10% SDS and 0.6 ml of water]. The bag was sealed, put into a water bath and kept at 42°C overnight. The prehybridization solution was replaced by 5 ml of hybridization solution (2.5 ml of 100% deionized formamide, 1.75 ml of 20 X SSPE, 0.5 ml of Denhardt's solution, 0.1 ml of 10mg/ml denatured salmon sperm DNA, 0.025 ml of 10% SDS and 0.125 ml of water) with a ^{32}P-labeled DNA probe. The bag was returned to the same water bath at 42°C for another 48 hours. After hybridization, the membrane was washed 4 times, for 10 minutes each, in 100 ml of 2 X SSC and 0.1% SDS. Then it was washed once in 200 ml of 1 X SSC and 0.1% SDS and kept in

200 ml of 1 X SSC and 0.1% SDS at 68°C for one hour. The membrane was placed onto a piece of Whatman 3 MM paper and dried at room temperature. Autoradiography with X-ray film (Kodak XAR5) with an intensifying screen (CRONEX, F1-929, E.I.duPont de Nemours & Co.) at -70°C was performed overnight.

(4) Dot-blot hybridization

A single bacterial colony was incubated in 10 ml of liquid LB medium plus 40 μg/ml ampicillin at 37°C overnight. Chloramphenicol was added to a final concentration of 50 μg/ml to amplify the plasmid and the culture was incubated at 37°C for 5 hours with shaking. One ml of the culture was transferred to an Eppendorf centrifuge tube and the cells were collected by centrifugation for 2 minutes. The cell pellet was dried and resuspended in 100 μl of freshly prepared lysozyme solution (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH8.0, 4 mg/ml lysozyme). Incubation was performed at room temperature for 5 minutes, followed by addition of 200 μl of fresh lysis solution (0.2N NaOH, 1% SDS). The mixture was vortexed gently and kept on ice for 5 minutes. The solution was neutralized by adding 150 μl of cold potassium acetate (pH4.8). After vortexing gently, it was put on ice for 5 minutes. After centrifugation at 4°C for 5 minutes in an Eppendorf centrifuge the supernatant was transferred to a clean Eppendorf tube and mixed with 45 μl of 3M NaOAc (pH was adjusted to 8.0 with glacial acetic acid) and 1 ml of 95% ethanol. The DNA was precipitated at

-70°C for 45 minutes. The DNA pellet was collected by centrifugation at 4°C for 15 minutes in an Eppendorf centrifuge and dried under vacuum. The pellet was dissolved in 10 µl of TE buffer. Finally, 3 µl of the DNA solution was spotted onto a piece of Biodyne transfer membrane. After spotting all the samples, the membrane was baked in an oven at 80°C for 2 hours. Prehybridization, hybridization and washing were the same as in colony hybridization procedures.

2.5 Transcription of RNA *in vitro*

The pDX1 and pDX4 plasmid DNAs, containing a cDNA copy of PSTV with opposite orientations in pSP64 and pSP65 (see the physical maps in Fig. 10), were digested with EcoRI (Pharmacia) and HindIII (Pharmacia) restriction endonucleases, respectively. The linearized DNA was precipitated by ethanol precipitation at -70°C and dissolved in DEPC (diethylpyrocarbonate) treated water prepared according to Maniatis *et al.* (1982) at a concentration of about 0.5 µg/µl.

The reaction mixture consisted of the following: 2 µl of 10 X SP6 transcription buffer (0.4M Tris-HCl, pH7.5, 0.06M $MgCl_2$, 0.02M spermidine), 0.2 µl of 1M DTT (dithiothreitol), 4 µl of 2.5mM rNTP, 1 µl of RNase inhibitor (Promega Biotec., 30 unit/µl), 4 µl of the linearized DNA template (about 2 µg), 5 µl of α-^{32}P UTP (Amersham Canada Limited, Cat. No. N.10203, 10 mCi/ml, about 3000 Ci/mmol), 2 µl of DEPC treated water and 2 µl of SP6

RNA polymerase (New England Nuclear, 7 unit/μl). The reaction was incubated at 37°C for one hour. After the reaction, 20 μl of deionized formamide was added and the mixture was kept at 95°C for 5 minutes to denaturate the RNA transcripts. The samples were loaded onto a 5% polyacrylamide denaturing gel with 8M urea.

In *in vitro* transcription studies where the infectivity of the RNA transcripts were going to be examined, radioactive α-^{32}P UTP was not used in the reaction mixture. After the reaction, the solution was directly extracted with an equal volume of phenol (saturated with TNE) followed by extraction with ether. The nucleic acids were ethanol precipitated and the pellet was dissolved in 50 μl of inoculation buffer (10mM Tris-HCl, pH8.0, 10mM EDTA).

2.6 Purification of RNA from 5% polyacrylamide gel

After electrophoresis, the RNA transcripts or PSTV RNA bands were located by UV illummination. They were excised from the gel, and added to 200 μl of TE buffer in Eppendorf tubes. A glass rod was used to homogenize the gel slices and 0.5 ml of phenol satuated with TE buffer was added immediately. The mixture was kept at room temperature for half an hour, the aqueous phase was collected by centrifugation for 2 minutes and extracted with 200 μl of ether to remove any trace of phenol. The solution was kept at 4°C overnight, then pelleted by centrifugation at 8,000 X *g* for 15 minutes. The supernatant was transferred into a

clean Eppendorf tube and 10 µl of yeast tRNA was added, followed by ethanol precipitation of the RNA at -20°C overnight. The RNA pellet was dissolved in an appropriate volume of inoculation buffer.

2.7 Infectivity tests

The tomato cultivar Rutgers, kindly provided by Drs. D. K. Lakshman and C. Hiruki, Department of Plant Science, University of Alberta, was used as a host for infectivity tests. The carborundum-dusted plants at the two-leaf stage (10 days after germination) were inoculated by mechanical inoculation with the RNA samples at a concentration of 1 µg of the transcribed RNA per plant, except for inoculation by PSTV-infected leaf juice (the supernatant solution after homogenizing PSTV infected-leaves). For negative controls (healthy plants), the leaves were rubbed with inoculation buffer. Five minutes after ~~the~~ inoculation, the leaves were rinsed with autoclaved water. For the development of disease, the plants were kept in a greenhouse at 26-28°C for at least four weeks under natural light (14 hours per day).

2.8 Detection of PSTV by nucleic acid spot hybridization

This protocol was performed according to the method of Owens and Diener (1984) with slight modifications.

Half a gram of fresh tomato leaves were collected into an Eppendorf tube. Then, 0.75 ml of the grinding buffer (0.5M NaOAc-HAc, pH6.0, 10mM $MgCl_2$, 20% ethanol, 3% SDS, 1M

NaCl and 0.05% bromophenol blue) was added. The sample was ground with a grinder with a 10N shaft (Terochem Laboratories LTD., Ultra-Turrax, drive T 18/10). After grinding, the tube was vortexed briefly and incubated at 37°C for 5 minutes. After the addition of 200 µl of chloroform, the mixture was vortexed and held on ice until all the tubes were prepared. The samples were centrifuged at room temperature for 2 minutes in an Eppendorf centrifuge. A piece of Biodyne transfer membrane (Pall Ultrafine Filtration Corporation, P/N BNNG2225) was wetted with water, then equilibrated with two changes of 20 X SSC and dried in an oven at 60°C. Five µl aliquots of clarified sap were spotted on the treated membrane. After all samples were spotted on the membrane, the membrane was baked at 80°C for 2 hours in an oven, then placed into a plastic bag with 25 ml of prehybridization solution (8 ml of deionized formamide, 2 ml of 1.8M NaCl-0.168M sodium cacodylate-0.032M cacodylic acid-10mM EDTA, 0.2 ml of 10% SDS, 1 ml of 2% bovine serum albumin-Ficoll-polyvinylpyrrolidone solution, 0.4 ml of 20 mg/ml yeast RNA, 2 ml of 10% glycine, and 6.4 ml of water). The bag was sealed and kept in a water bath at 42°C for 2 hours. Prehybridization solution was replaced by hybridization solution (8 ml of deionized formamide, 2 ml of 1.7M NaCl-0.168M sodium cacodylate-0.032M cacodylic acid-10 mM EDTA, 0.2 ml of 10% SDS, 1 ml of 2% bovine serum albumin-Ficoll-polyvinylpyrrolidone solution, 0.4 ml of 20 mg/ml yeast RNA, 4 ml of 50% Dextran sulfate, 0.44 ml of

water and ^{32}P-labeled probe in a small volume of TE to which an equal volume of deionized formamide had been added). The hybridization was carried out at 55°C for 48 hours in a water bath. After hybridization, the membrane was washed with 100 ml of 2 X SSC plus 0.5% SDS four times at 55°C, 15 minutes each. It was then washed with 100 ml of 0.2 X SSC plus 0.5% SDS twice at 55°C, 15 minutes each. The membrane was transferred onto Whatman 3 MM paper and dried at room temperature followed by autoradiography with X-ray film (Kodak XAR5) and an intensifying screen (CRONEX, F1-929, E.I.du Pontde Nemours & Co.) at -70°C for 2 or3 days.

2.9 Detection of PSTV RNAs by extraction of total nucleic acids from PSTV infected plants and polyacrylamide gel electrophoresis

This protocol is based on Morris' procedure (Morris and Smith, 1977) with modifications.

One gram of terminal tip tissue of tomato leaves was ground in a solution containing 0.5 ml of extraction buffer (0.2M glycine, 0.1M $NaHPO_4$, 0.6M NaCl, 1% SDS adjusted to pH9.5 with 5M NaOH), 1 drop of mercaptoethanol, 2 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline and 2 ml of chloroform-pentanol (25:1 v/v). Extraction was accomplished by mixing all the reagents with the tissue and homogenizing the mixture for a few seconds at room temperature in a grinder with a 10N shaft (Terochem Laboratories LTD., Ultra-Turrax, drive T 18/10). The

homogenate was centrifuged at 12,100 X *g* for 15 minutes at 4°C. The clear upper aqueous phase was collected after centrifugation. An equal volume of 4M LiCl was added to the supernatant to make a final concentration of 2M LiCl in the solution. This solution was kept at 4°C overnight, and then centrifuged at 7,700 X *g* for 15 minutes at 4°C. The clear supernatant solution was saved. One-tenth volume of 3M sodium acetate (pH8.0) and 2.5 volumes of 95% ethanol were mixed with the supernatant and kept at -20°C overnight. The solution was centrifuged at 7,700 X *g* for 15 minutes at 4°C and the pellet was collected.

At this stage, the sample was ready for electrophoresis. In order to eliminate some cellular RNAs, especially 9S RNA which is the major source of viroid contamination seen in gel electrophoresis, treatment by isopropanol precipitation was carried out as follows:

The pellet was dissolved in 0.5 ml of 0.3M sodium acetate (pH7.0) and then mixed with 0.54 volumes of isopropanol. The mixture was kept at -20°C for a few minutes, then centrifuged at 7,700 X *g* for 10 minutes at 4°C. The supernatant was collected and stored at 4°C. The precipitate was resuspended in 0.3M sodium acetate (pH7.0) and mixed with 0.54 volumes of isopropanol. The mixture was kept at -20°C and centrifuged as above. The supernatant was combined with the previously obtained supernatant and mixed with 2.5 volume of 95% ethanol. The mixture was kept at -20°C overnight. The pellet was obtained by centrifugation

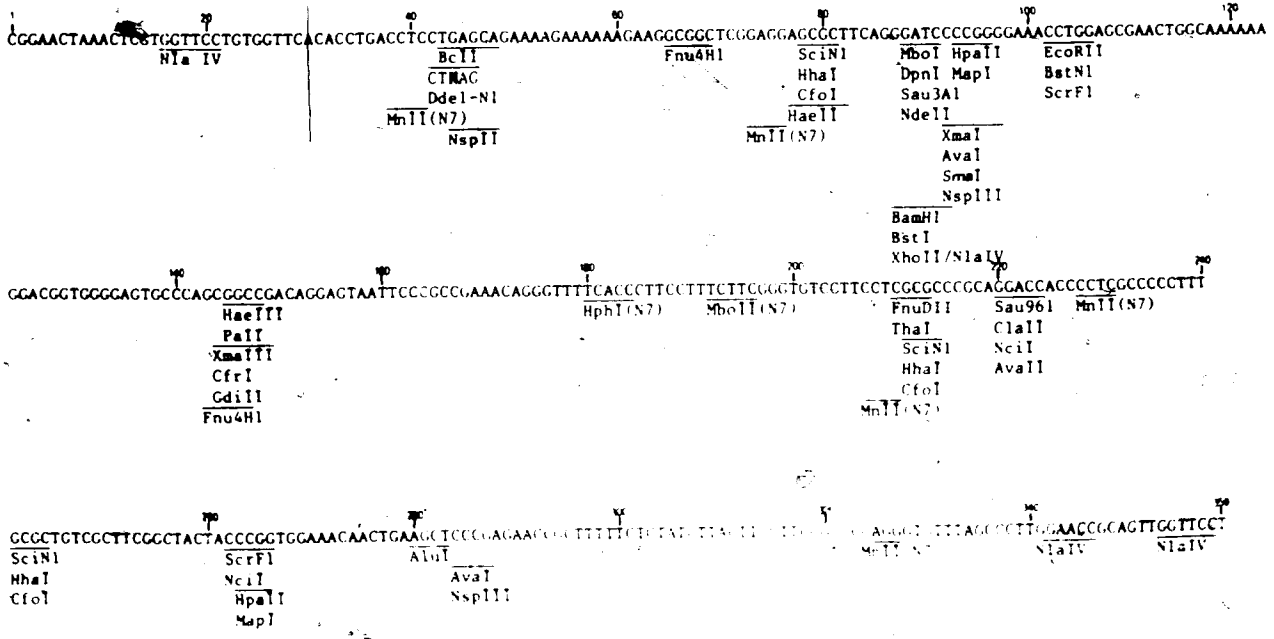
at 7,700 x g for 20 minutes at 4°C and dried under vacuum. TE buffer (100 µl) was added to resuspend the pellet. For electrophoresis, a 50 µl aliquot of the solution was mixed with an equal volume of deionized formamide containing bromophenol blue and xylene cyanol and loaded on a 5% polyacrylamide gel containing 8M urea. Following electrophoresis, the gel was stained in 1 µg/ml ethidium bromide in water for about 30 minutes and observed by UV illumination.

3. Results

3.1 The location of the restriction endonuclease recognition sites in the PSTV cDNA sequence

It is very useful to know the location of the restriction endonuclease recognition sites in the PSTV cDNA sequence in order to perform genetic manipulations *in vitro*. Gross *et al*. (1978) determined the primary RNA sequence of PSTV by RNA fingerprinting techniques followed by sequencing of the RNA fragments. We subjected the corresponding cDNA sequence to a computer search in order to identify useful restriction endonuclease cleavage sites. The results shown in Fig. 3 indicate that a number of restriction enzyme cleavage sites were found. Unfortunately, most of them are not commonly used in cloning procedures. Several commonly used restriction enzyme cleavage sites, such as BamH1, SmaI, AvaI, XmaI, etc., were found in a small region of the cDNA from nucleotides numbers 80 to 100, which is closed to a region where mutations for most of the naturally occurring PSTV mutants are located.

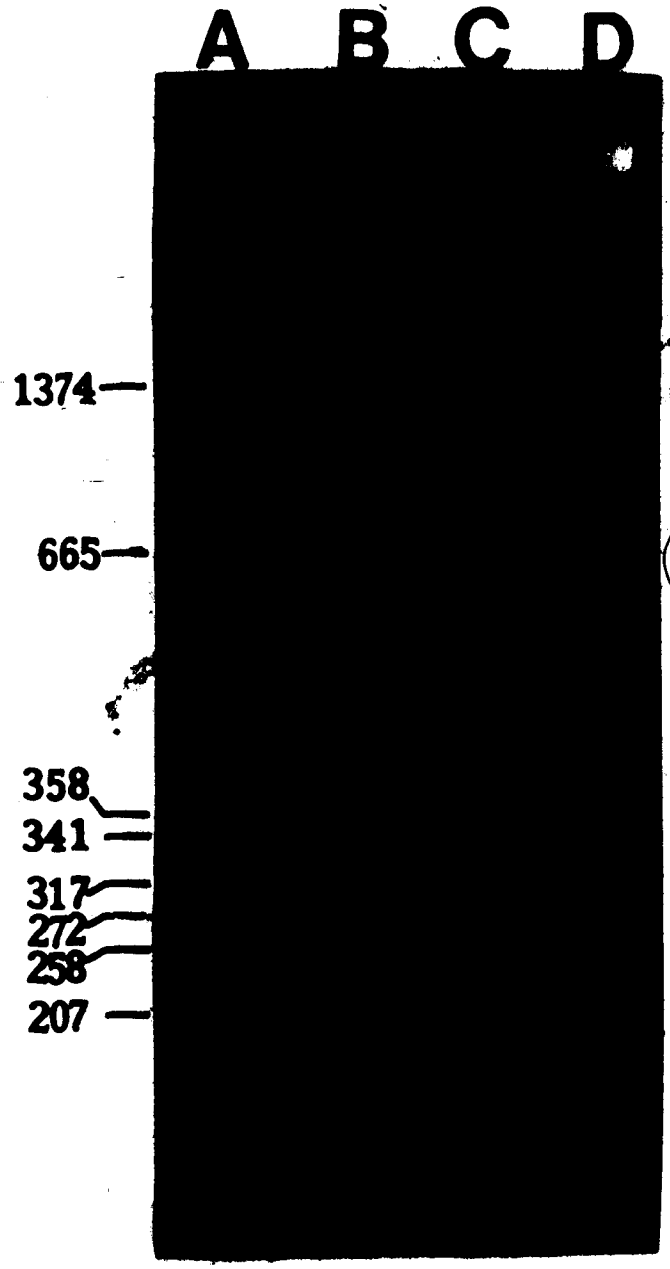
Fig. 3. The location of restriction endonuclease recognition sites in the PSTV cDNA sequence. The polarity of this cDNA sequence is the same as PSTV (+) RNA sequence determined by Gross *et al.* (1978).


CGGAACTAAACTCGTGGTTCCTGTGGTTCACACCTGACCTCCTGAGGAGAAAAGAAAAAAGAAGGCGGCTCGGAGGAGCCCTTCAGGGATCCCCGGGGAAAACCTGGAGCGAACTGGCAAAAAA
NIa IV
BclI
CTNAG
Dde1-N1
MnlI(N7)
NspII
Fnu4H1
SciN1
HhaI
CfoI
HaeII
MnlI(N7)
MboI
DpnI
Sau3A1
NdeII
HpaII
MapI
EcoRII
BstN1
ScrF1
XmaI
AvaI
SmaI
NspIII
BamHI
BstI
XhoII/N1aIV
GGACGGTGGGGAGTGCCCAGCGGCCGACAGGAGTAATTCCCGCCGAAACAGGGTTTTCACCCTTCCTTTCTTCGGGTGTCCTTCCTCGCGCCCCGCAGGACCACCCCTCGCCCCCTTT
HaeIII
PaII
XmaIII
CfrI
GdiII
Fnu4H1
HphI(N7)
MboII(N7)
FnuDII
ThaI
SciN1
HhaI
CfoI
MnlI(N7)
Sau961
ClaII
NciI
AvaII
MnlI(N7)
GCGCTCTCGCTTCGGCTACTACCCCGTGGAAACAACTGAAGCTCCCGAGAACG
SciN1
HhaI
CfoI
ScrFI
NciI
HpaII
MapI
AluI
AvaI
NspIII
NlaIV
NlaIV

3.2 Characterization of a full length cDNA clone of PSTV

The clone pAV401 containing a full length, 359 base pairs long, cDNA of PSTV cloned at the BamH1 site of the plasmid pBR322 was originally supplied by Dr. Pieter Vos (Van Wezenbeck *et al.*, 1982). The PSTV cDNA insert was isolated from the clone pAV401 by digestion with BamH1 enzyme. The samples were analyzed by electrophoresis in 5% polyacrylamide gels containing 8M urea followed by staining with 0.5 μg/ml of ethidium bromide in TE buffer. The plasmid pBR322 digested with Sau3A1 enzyme was used as a molecular size marker because one of the 22 resulting fragments is 358 nucleotides in length. As seen under UV light, as evident in Fig. 4, a DNA band from plasmid pAV401 (lane A) migrates closely to the 358 base pair band in the molecular size marker (lane B). No such band is visible in the BamH1 digestion product of the parent plasmid pBR322 (lane C). This confirms that the clone pAV401 contains an insert 359 nucleotides in length.

Fig. 4. Determination of the size of the PSTV specific cDNA insert in the pAV401 recombinant clone by gel electrophoresis in a 5% polyacrylamide gel with 8M urea. Lane A: pAV401 DNA digested with BamH1 enzyme. Lane B: pBR322 digested with Sau3A1 enzyme. Lane C: pBR322 digested with BamH1 enzyme. Lane D: undigested pBR322.

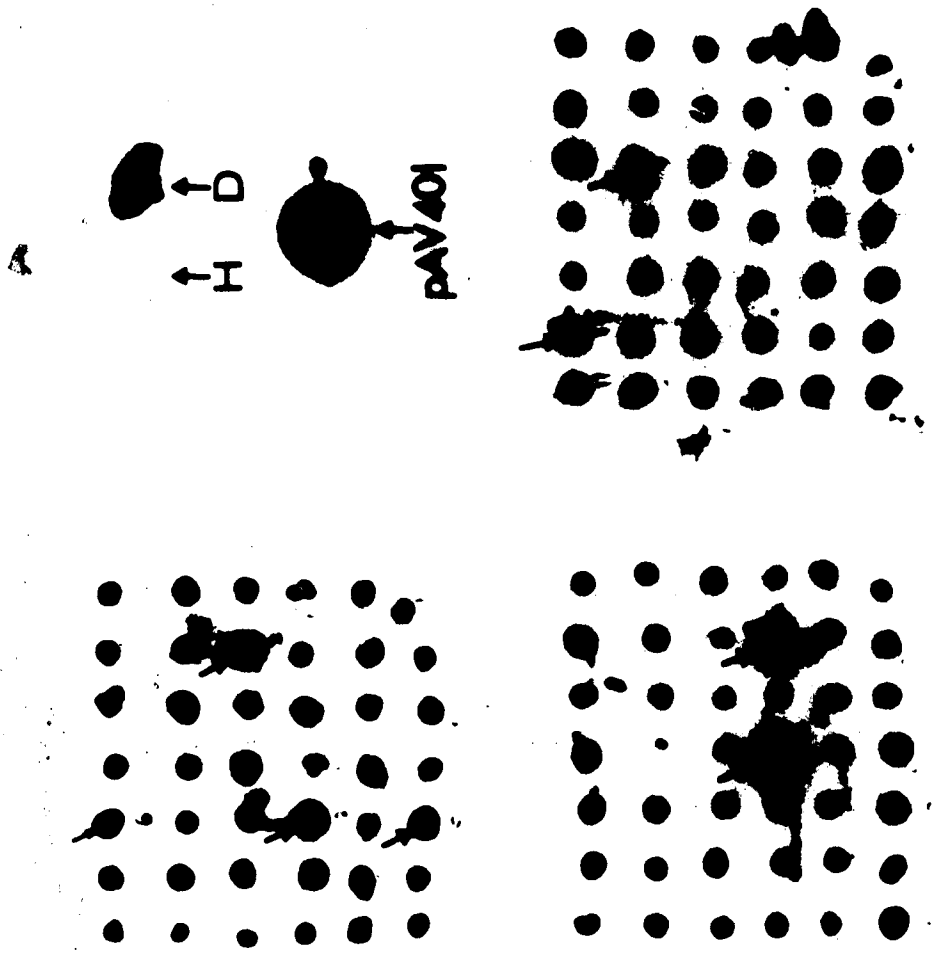

A
B
C
D
1374
665
358
341
317
272
258
207

3.3 Subcloning of PSTV specific cDNA fragment into pSP6 plasmid vectors

(1) Subcloning of PSTV specific cDNA fragment from pAV401 into a pSP64 vector

The BamH1 DNA fragment with 359 base pairs from pAV401 was obtained by extraction after gel electrophoresis in 1.5% low melting point agarose. It was ligated into the pSP64 vector already digested with BamH1 and dephosphorylated with bacterial alkaline phosphatase. The recombinant plasmid DNA was then used to transform *E. coli* HB101 strain. Thousands of bacterial colonies were obtained by plating the transformants on LB plates containing ampicillin. Colony hybridization was performed to screen for colonies containing PSTV cDNA inserts. One hundred and sixty eight colonies were randomly selected and transferred onto Biodyne transfer membranes. The PSTV specific cDNA fragment from pAV401 was radiolabelled by nick translation. Nucleic acid preparations isolated from healthy tomato plants and PSTV-infected plants were also spotted onto the membrane as controls. The result of the hybridization experiment is shown in Fig. 5. The nucleic acid preparation isolated from healthy tomato plants gave no hybridization signal, whereas the nucleic acids isolated from the PSTV-infected plants was positive for the PSTV sequence, indicating that the probe and the hybridization conditions used were specific for PSTV sequence. By comparison with the hybridization signal of the positive control (plasmid pAV401), 8 colonies were chosen.

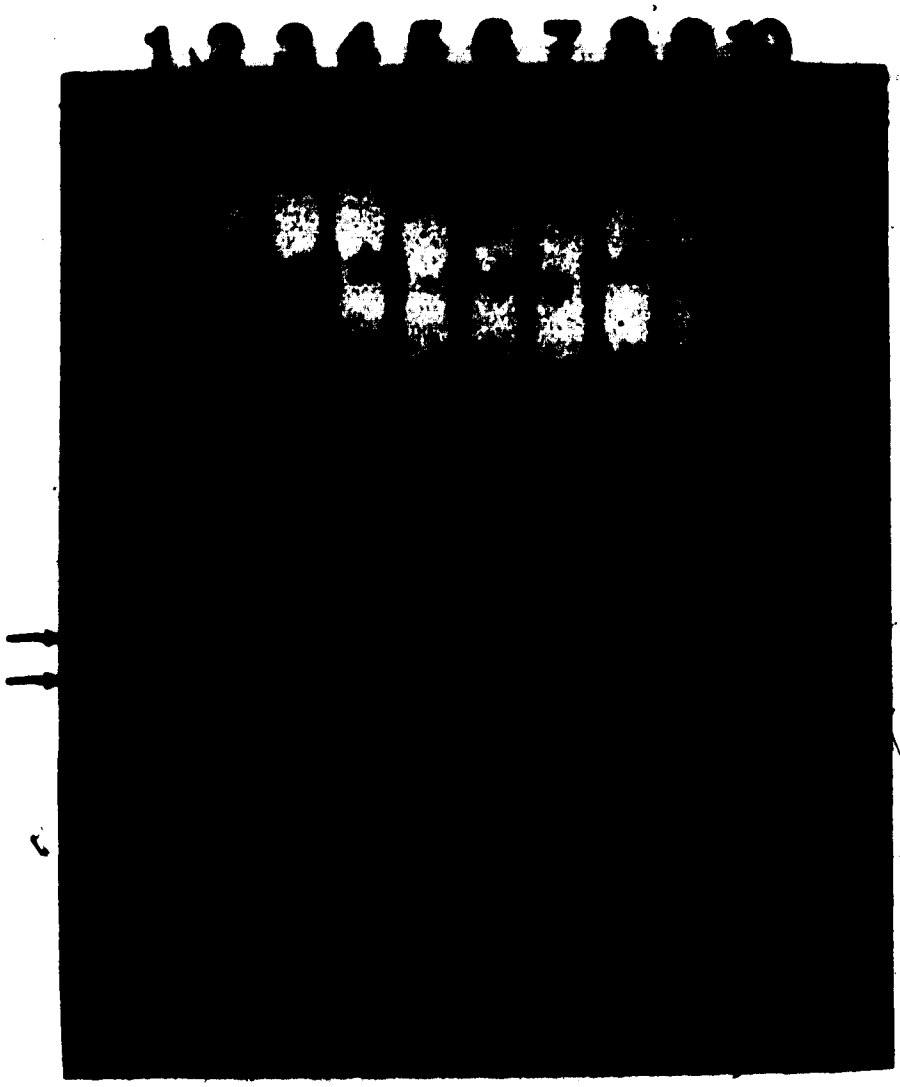
Fig. 5. Colony hybridization of bacterial colonies obtained by subcloning of PSTV cDNA into a pSP64 vector. The probe was made by nick translation of PSTV specific cDNA fragment. pAV401: the plasmid pAV401 was spotted on the membrane as a positive control. H: the nucleic acid preparation isolated from healthy tomato plants. D: the nucleic acid preparation isolated from PSTV-infected tomato plants. Arrows indicate the 8 bacterial colonies selected as positive for hybridization signals.


D
I
PAVION

(2) Confirmation of the presence of the inserts in the recombinant pSP64 and determination of the forms of the inserts

Plasmid DNAs from the eight recombinant clones were prepared by the CsCl-EtBr centrifugation method. To confirm that these clones do contain inserts and to distinguish between monomeric and multimeric DNA forms, the plasmid DNAs were double-digested with EcoRI and HindIII enzymes (see Fig. 1). If the clone contained the monomeric cDNA insert, the released DNA fragment (409 base pairs in length) should be slightly larger than the PSTV cDNA because the DNA fragment produced by the double-digestion contains extra polylinker sequences from the vector. If the insert exists in the multimeric form, the DNA band produced by double-digestion should be multiples of 359 base pairs plus the extra sequences. After enzyme digestion followed by gel electrophoresis analysis in 1.5% agarose, two clones were found to contain the PSTV cDNA copy. Both inserts existed in the monomeric form (Fig. 6).

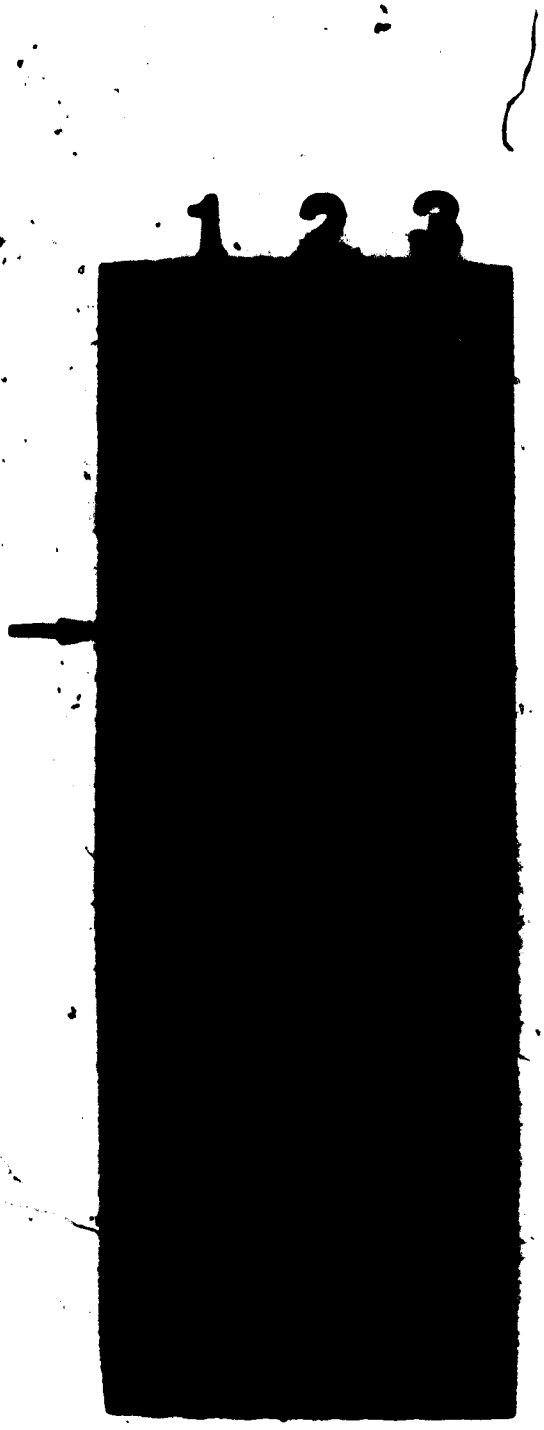
Fig. 6. Determination of the presence of the inserts during subcloning of PSTV cDNA into a pSP64 vector. Lane 1: pAV401 DNA digested with BamH1 enzyme. Lane 2-9: the recombinant plasmid DNAs digested with EcoRI and HindIII enzymes. Lane 10: pSP64 plasmid DNA digested with EcoRI and HindIII enzymes. After digestion, the DNA fragments were electrophoresized in 1.5% agarose gel. The DNA bands were visualized by staining with EtBr. The arrows indicate the positions of the inserts on the gel.



(3) Determination of the orientation of the inserts in the recombinant clones

The orientation of the cDNA inserts in the two recombinant clones were determined as follows. A SmaI restriction endonuclease cleavage site was found next to the BamH1 cleavage site on PSTV cDNA and was also presented on the polylinker of the plasmid vector pSP64. If the PSTV cDNA insert was in the (+) orientation, the recombinant clone should yield a DNA fragment of about 360 base pairs long upon digestion by SmaI. If the PSTV cDNA insert was in the (-) orientation, a very small DNA fragment (around 10 base pairs long) will be produced after SmaI digestion. Based on this calculation, the two recombinant plasmids were digested with SmaI enzyme followed by electrophoresis in a 5% polyacrylamide gel with 8M urea. The result is shown in Fig. 7. Clone #1 (lane 2) contains PSTV specific insert at the approximate size of 360 base pairs. Clone #2 (lane 3) contains two bands, which may have been generated by re-arrangment to result in a new SmaI cut site on the vector. From the result, it was concluded that the recombinant plasmid (Clone #1) contained the PSTV cDNA insert in a (+) orientation. Clone #2 was not used for further experiments.

Fig. 7. Determination of the orientation of the cDNA inserts in the recombinant clones in a 5% polyacrylamide gel ~~with~~ 8M urea. Lane 1: pAV401 DNA digested with BamH1 enzyme. Lane 2: the recombinant plasmid DNA clone #1 digested with SmaI enzyme. Lane 3: the recombinant plasmid DNA clone #2 digested with SmaI enzyme. The arrow indicates the positions of the inserts on the gel.



(4) Subcloning of PSTV specific cDNA in the opposite orientation

In order to obtain a PSTV cDNA inserted in the opposite orientation, the pSP65 plasmid was used. This vector is essentially the same plasmid as pSP64, but the orientation of the polylinker is reversed (Fig. 1). Since the EcoRI and HindIII restriction sites are present in the polylinker but not in the PSTV cDNA, these cleavage sites were used to construct the recombinant plasmids. The recombinant plasmid clone #1 and pSP65 were both digested with EcoRI and HindIII enzymes. The DNA fragment containing the PSTV cDNA from clone #1 was cloned into pSP65. After ligation and transformation, a total of 40 colonies were obtained. Dot-blot hybridization was used to screen these colonies. The PSTV cDNA from pAV401 plasmid was used as a nick translation probe. As shown in Fig. 8, six clones showing positive hybridization signals were selected. Plasmid DNAs isolated from the six clones were digested with BamH1 and analysed by gel electrophoresis. Four out of six contained the expected cDNA inserts (Fig. 9). The plasmid DNA in lane 2 was randomly selected as the recombinant clone containing PSTV cDNA in the (-) orientation to be used in further experiments. It was designated as pDX4. RNA transcribed from this clone would be indentical to PSTV (+) RNA. Clone #1 with the PSTV cDNA insert in the (+) orientation was designated as pDX1. RNA transcribed from this clone is the same polarity as PSTV RNA. Restriction endonuclease maps of

these two clones are shown in Fig. 10.

3.4 Transcription of PSTV (+) and (-) RNA from pDX1 and pDX4 *in vitro*

(1) *In vitro* transcription of PSTV (+) RNA in the presence of α-^{32}P UTP

In order to test the transcription *in vitro* using these plasmids as DNA templates, α-^{32}P UTP was used as a substrate for autoradiographic detection of RNA transcripts. The plasmid pDX1 was first linearized by digestion with the EcoRI enzyme which cleaves at the polylinker site downstream from the PSTV cDNA insert. Two μg of the linearized DNA recovered by ethanol precipitation were used as templates in each transcription reaction. Native PSTV RNA isolated from PSTV-infected plants was used as a marker. After *in vitro* transcription using SP6 RNA polymerase, samples were analysed in a 6% polyacrylamide gel with 8M urea and the gel was dried under vacuum. The results are shown in Fig. 11 (Fig. 11A shows a gel stained by ethidium bromide and Fig. 11B shows an autoradiograph of the dried gel).

These results show that, as expected, the correct RNA transcripts were obtained from pDX1 since a band appeared (lane 2) at a position close to the viroid RNA band (lane 1). The autoradiograph showed a RNA transcript band from pDX1. These results indicate that the plasmid DNA can serve as a template for SP6 RNA polymerase and correct transcripts can be obtained from pDX1.

Fig. 8. Dot-blot hybridization to screen for PSTV specific sequences during subcloning into a pSP65 vector. PSTV specific cDNA from clone pAV401 was nick translated and used as the hybridization probe. Arrows indicated the bacterial colonies exhibiting positive hybridization signals and being selected for further characterization.

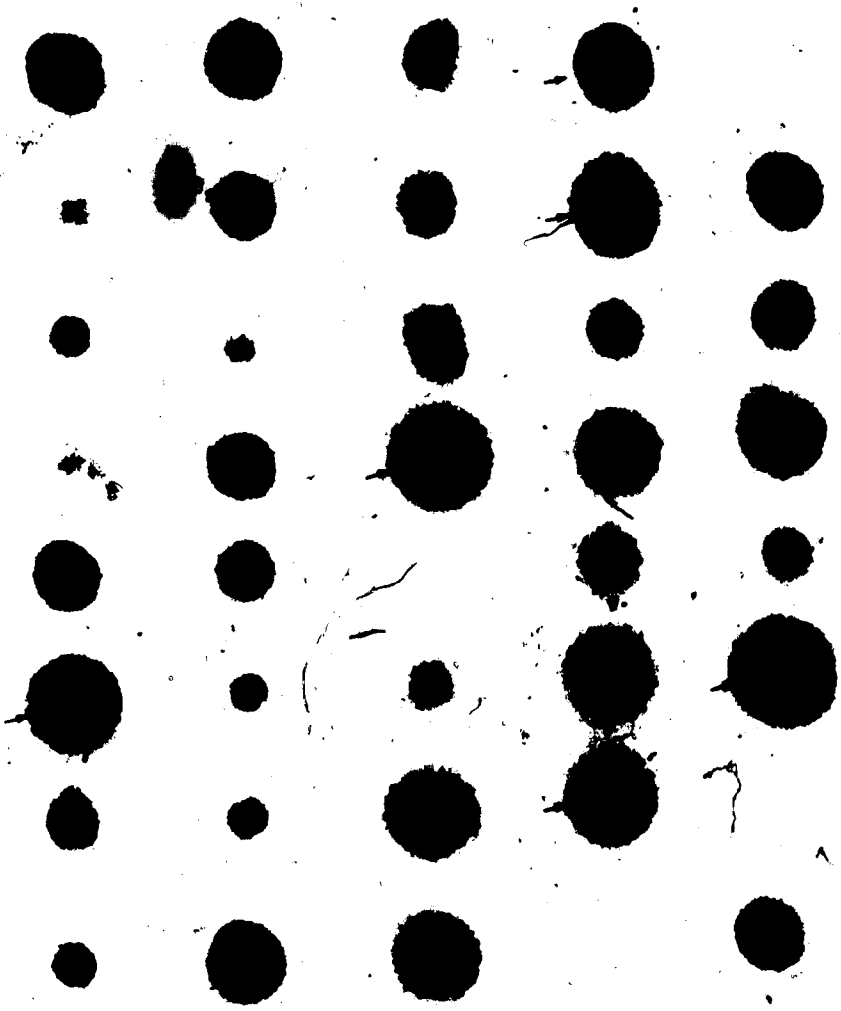


Fig. 9. Restriction endonuclease analysis of the recombinant clones obtained during subcloning into a pSP65 vector. Six clones exhibiting positive hybridization signals were selected from 40 colonies. The plasmid DNAs were digested with BamH1 enzyme and analysed by gel electrophoresis in a 1.5% agarose gel. The arrow indicates the PSTV cDNA fragments released from the recombinant plasmids.

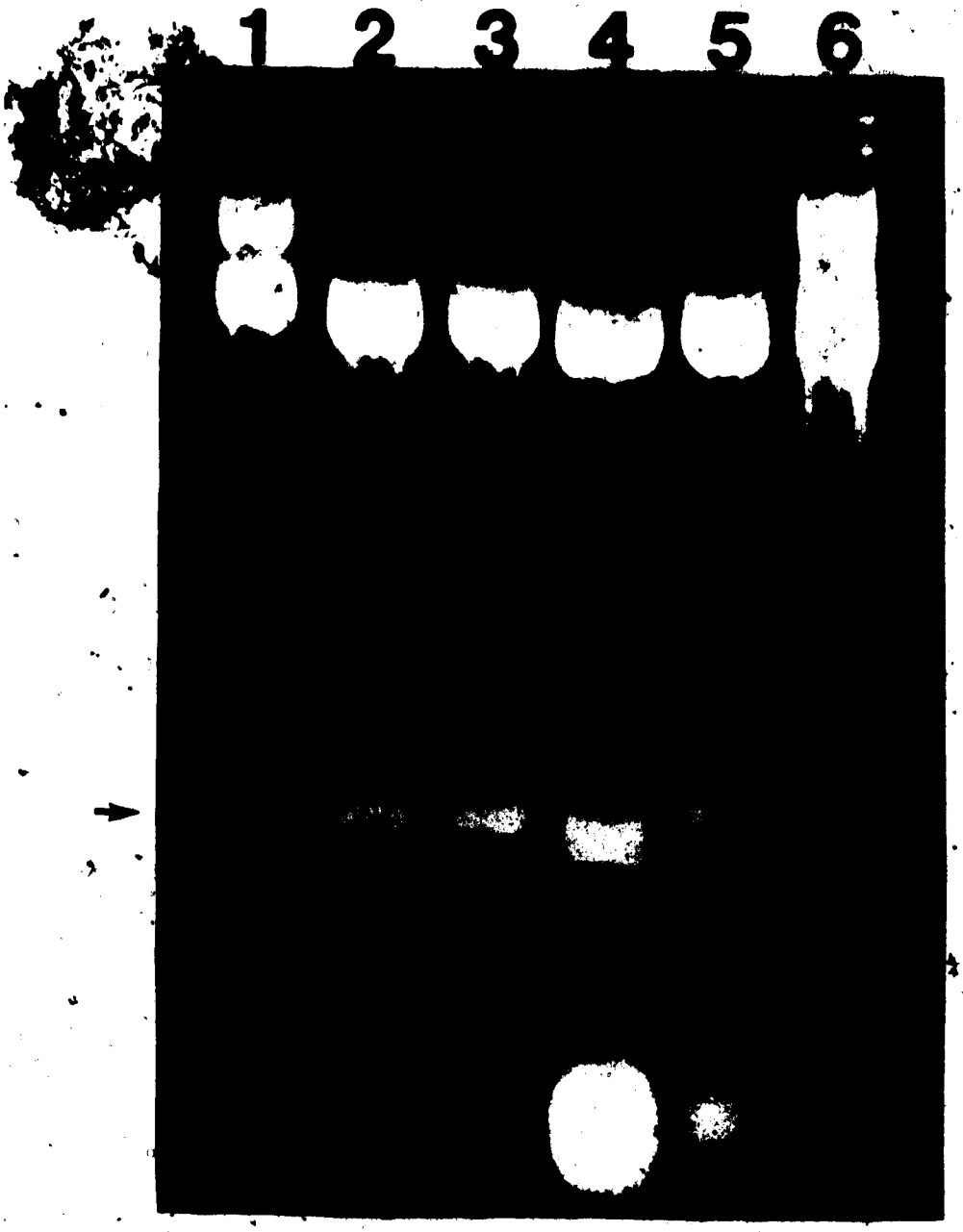
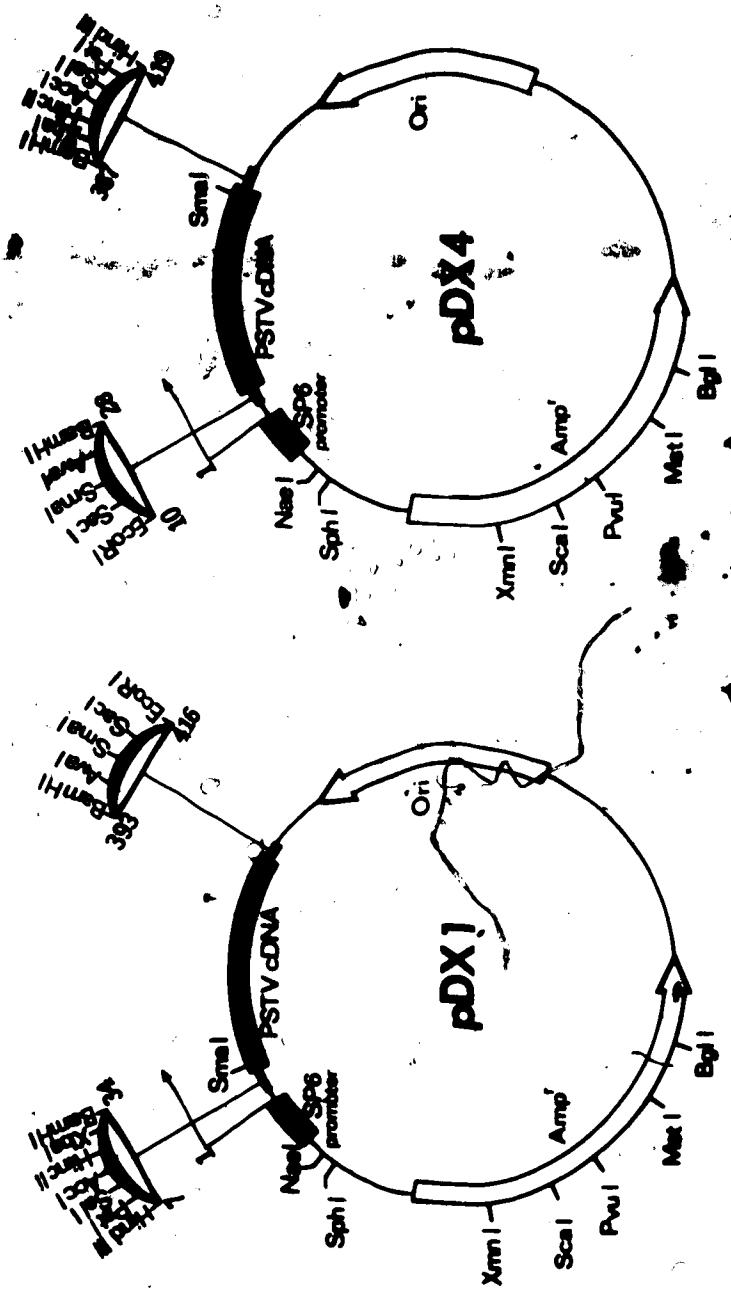

1 2 3 4 5 6

Fig. 10. Restriction maps of pDX1 and pDX4 recombinant plasmids. The PSTV cDNA insert in pDX1 is in the (+) orientation, whereas that in pDX4 is in the (-) orientation. The number 1 indicates the initiation site of RNA transcription for the SP6 promoter.


pDX4
Ori
PSTV cDNA
SP6 promoter
SmaI
NaeI
SphI
XmnI
ScaI
PvuI
MstI
BglI
Amp'
pDX1
Ori
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SP6 promoter
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PvuI
MstI
BglI
Amp'

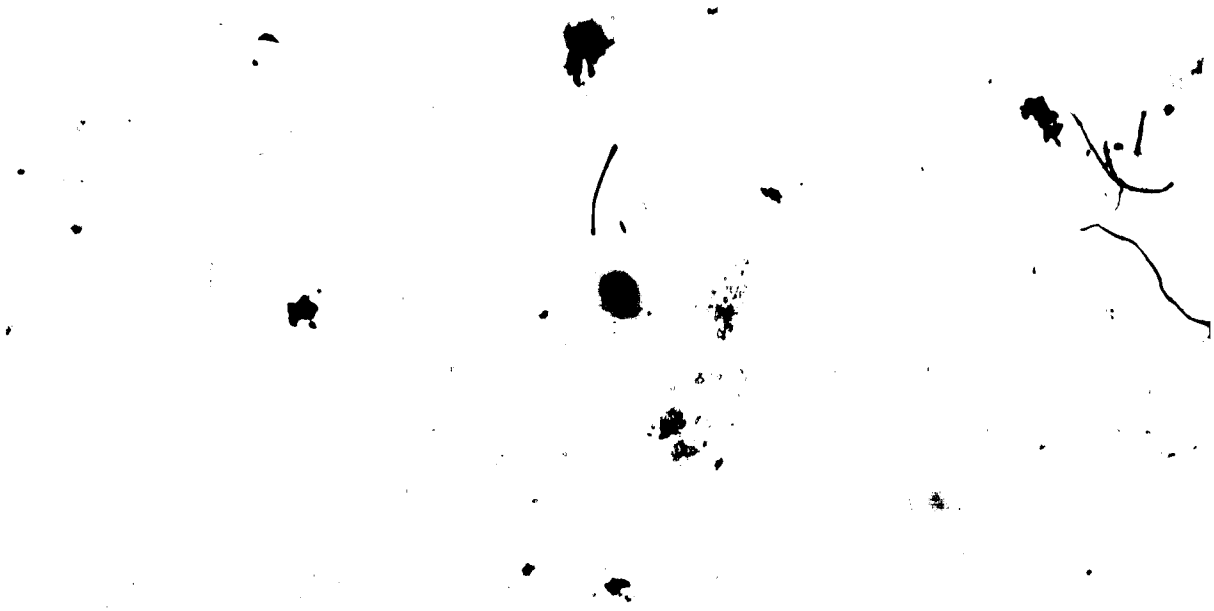
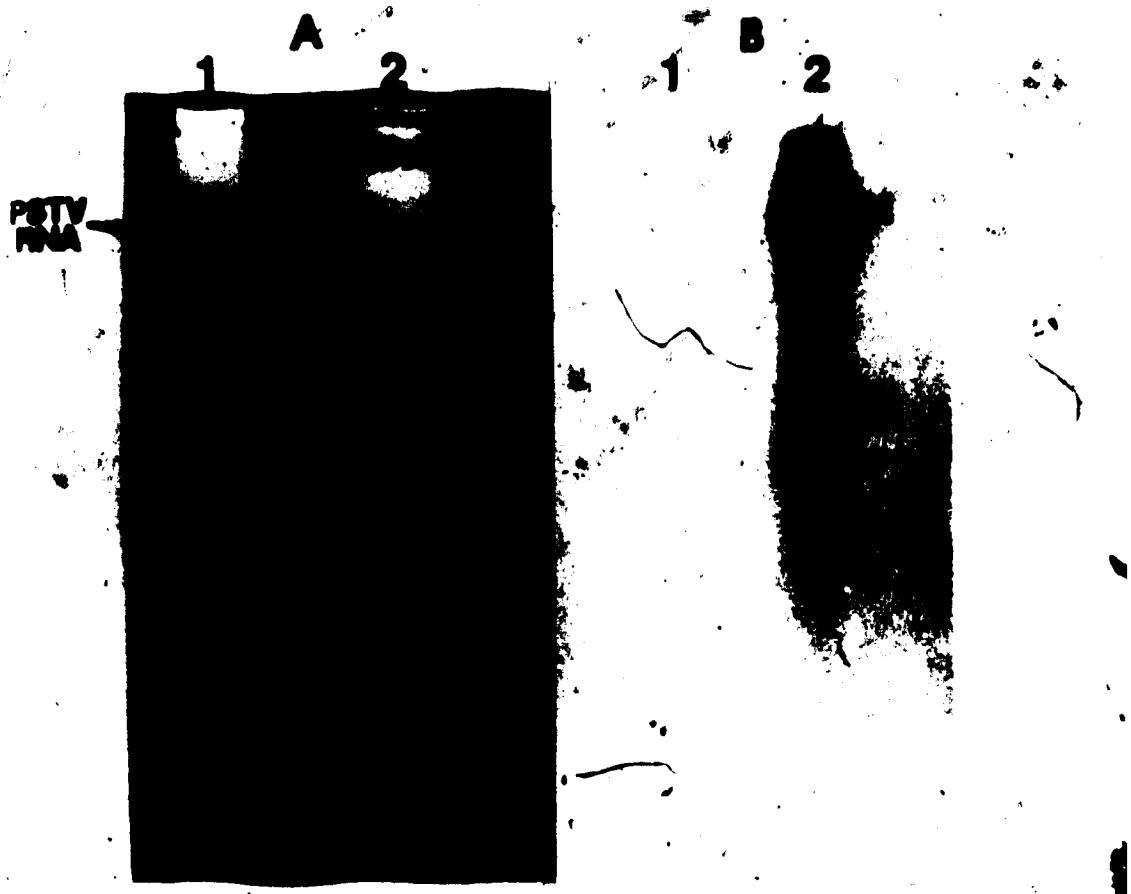


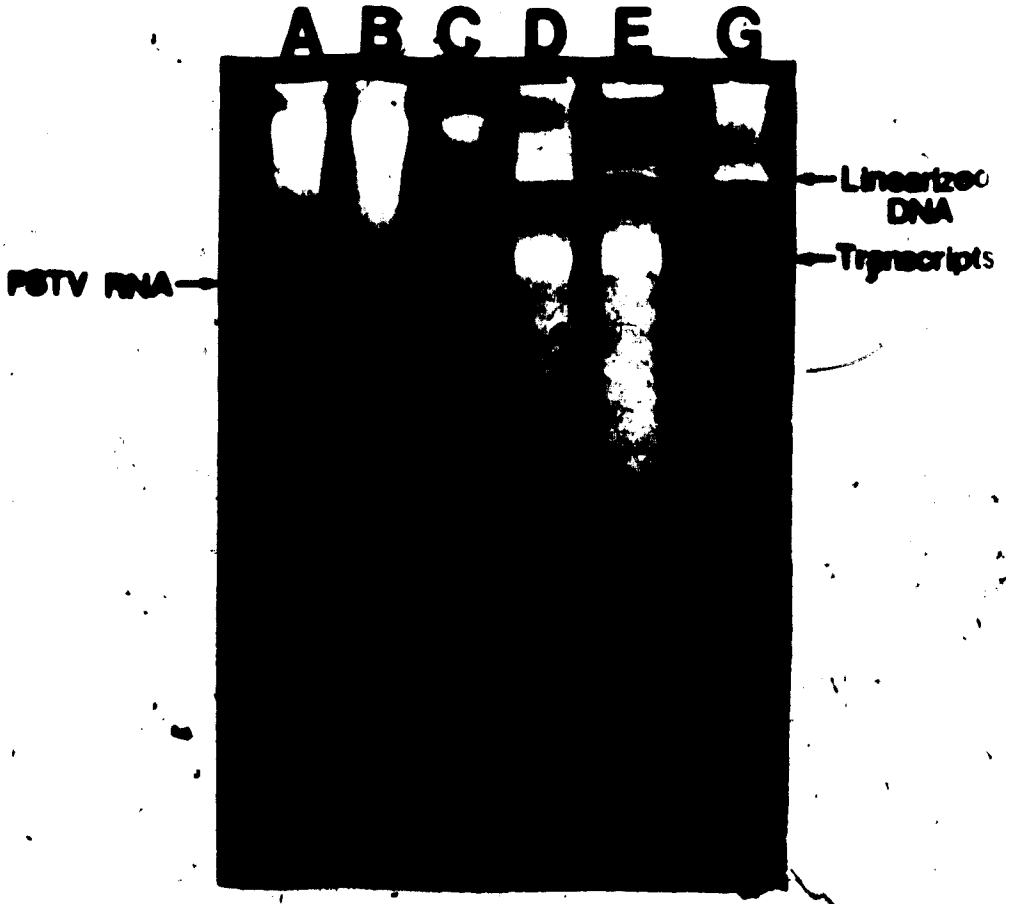
Fig. 11. *In vitro* transcription of pDX1 with α-^{32}P UTP. Fig. 11A: samples visualized under UV light after running in a 6% polyacylamide gel with 8M urea. Fig. 11B: autoradiograph of the dried gel. Lane 1: the nucleic acids isolated from PSTV-infected tomato plants. Lane 2: the nucleic acids transcribed from pDX1. Arrows indicate the positions of the RNA transcripts on the gel and the X-ray film.


A
1
2
PSTV RNA
B
1
2

(2) *In vitro* transcription of (+) and (-) RNAs without radioisotope labelling

From Fig. 11A, it can be seen that the (+) RNA transcripts migrated closely to the DNA band. To increase the resolution, 5% polyacrylamide gels rather than 6% were used to separate the RNA transcripts from the DNA templates. The pDX1 and pDX4 plasmids were linearized by EcoRI and HindIII enzymes, respectively. In the transcription reaction, no α-^{32}P UTP was added since the RNA transcripts were to be used for biological assays. As shown in Fig. 12, the transcripts can be clearly seen under UV light (lanes D and E). No such RNA is produced in the transcription reaction without the addition of SP6 polymerase (lane G). As expected, the transcribed RNAs were larger than the native PSTV RNAs because the transcripts contain extra sequences derived from the polylinker at both ends. Areas of the gel containing RNA bands from both transcripts and native viroid RNAs (lanes A and C) were excised. The RNAs were eluted and tested for infectivity. Similarly, non-denaturing 5% polyacrylamide gels were used to obtain non-denatured transcripts and PSTV RNA.

Fig. 12. *In vitro* transcription from linearized pDX1 and pDX4 DNA by SP6 RNA polymerase. The (+) RNA and (-) RNA transcripts were analysed by electrophoresis in a 5% polyacrylamide gel with 8M urea. Lanes A and C: the nucleic acid preparations isolated from PSTV-infected tomato plants. Lane B: the nucleic acid preparations isolated from uninfected tomato plants. Lane D: transcripts of pDX1 linearized with EcoRI enzyme. Lane E: transcripts of pDX4 linearized with HindIII enzyme. Lane G: linearized pDX plasmids without the addition of SP6 RNA polymerase.


A B C D E G
Linearized DNA
Transcripts
PSTV RNA

3.5 Infectivity tests

The transcripts and PSTV RNAs were extracted from the gel and infectivity tests were carried out on tomato plants as described in Materials and methods. Each of the nucleic acids, RNA transcripts, viroid RNAs and the plasmid DNAs which contain the PSTV monomers, was inoculated at 1 µg per plant. Typical symptoms of the disease were observed two weeks after inoculation with native PSTV RNA. In group 2 where plants were inoculated with a mixture of (+) RNA and pDX1 DNA, PSTV symptoms developed at three weeks after inoculation in one plant and six weeks after inoculation in two additional plants. One plant inoculated with (+) RNA transcripts isolated from a non-denaturing gel developed the symptoms at six weeks after inoculation. None of the healthy control plants (mock inoculated controls) ever developed the symptoms (see Table 1).

Table 2. Infectivity tests for PSTV and RNA transcripts

Nucleic acids, pDX1 or pDX4 as well as the (+) or (-) RNA transcripts, were inoculated at 1 μg per plant.

a: plasmid pDX1 and pDX4 DNAs were linearized by EcoRI or HindIII restriction endonucleases, respectively.

b: (+) RNA has the same sequence as PSTV RNA except for a small extra sequence at each end derived from the polylinker of the vector.

c: (-) RNA has the complementary sequence to PSTV RNA and also has a small extra sequence at each end derived from the polylinker of the vector.

Table 2. Infectivity Tests for PSTV and Transcripts

Group:	Plants inoculated with:	No. of infected plants/ No. of inoculated plants
1	pDX1 DNA[a]	0/10
2	(+) RNA[b] and pDX1 DNA	3/10
3	pDX4 DNA[a]	0/10
4	(-) RNA[c] and pDX4 DNA	0/10
5	(+) RNA isolated from non-denaturing gel	1/10
6	(-) RNA isolated from non-denaturing gel	0/10
7	(+) RNA isolated from denaturing gel	0/10
8	(-) RNA isolated from denaturing gel	0/10
9	Native PSTV RNA isolated from gel	10/10
10	Leaf juice from PSTV-infected plants	10/10
11	Total nucleic acids extracted from PSTV-infected plants	10/10
12	Control	0/5

3.6 Spot hybridization for detection of viroid RNA in inoculated plants

Spot hybridization experiments were carried out to verify the presence of PSTV RNA using a nick-translated PSTV cDNA probe. Juice from leaf samples was collected four weeks after the inoculation and 5 µl aliquots were spotted on a Biodyne transfer membrane. The result of the hybridization experiment is shown in Fig. 13. Leaf juice from all of the plants infected by native PSTV RNA showed positive hybridization signals (groups 9, 10 and 11). Leaf juice from all of the healthy control plants (group 12, mock infected) showed negative results. As expected, in the plants inoculated with (+) RNA and pDX1 DNA (group 2), the one plant showing early disease symptoms presented a strongly positive hybridization signal (f,2). Another plant which developed the disease symptoms later exhibited a weak hybridization signal (h,2). In group 5, one plant which was inoculated with (+) RNA isolated from a non-denaturing gel and developed the disease symptoms late also gave a weak hybridization signal (g,5). In no case did juice from the plants inoculated with pDX1 DNA (group 1) and pDX4 DNA (group 3) hybridize with the probe. Moreover, results from all of the plants inoculated with either (-) RNA and pDX4 DNA (group 4), or (-) RNA transcripts (groups 6 and 8) were negative. The plants inoculated with (+) or (-) RNA transcripts isolated from denaturing gels (groups 7 and 8) gave negative hybridization signals. These observations,

together with the results from the infectivity tests, suggest that (-) monomeric RNA of PSTV is not infectious.

At six weeks after inoculation, one plant inoculated with (+) RNA (group 5) and two plants inoculated with (+) RNA and pDX1 DNA (group 2) developed the disease symptoms. The leaf juices extracted from the diseased plants were collected and spotted on the membrane. All three of them showed positive hybridization signals [see Fig. 14(a)]. The RNA transcripts from pDX1 and pDX4 DNA carried extra sequences at both ends. It would be interesting to know whether or not the extra sequences persisted during replication of the PSTV RNA genome. The pSP64 plasmid without PSTV inserts was used as a probe to test for hybridization with the spotted juice samples. The results are shown in Fig. 14(b). In contrast to the hybridization obtained using PSTV cDNA as a probe, there was no positive hybridization signal when pSP64 plasmid DNA was used as a probe. This suggests that the extra sequences were excised during the replication of PSTV RNA.

Fig. 13. Spot hybridization to detect the presence of PSTV RNA in inoculated plants. A nick-translated PSTV cDNA was used as the hybridization probe. Four weeks after inoculation, leaf juice was extracted from these plants (10 plants for each group, a to j) and 5 μl aliquots of the juice from each plant was spotted as a single dot on the membrane.

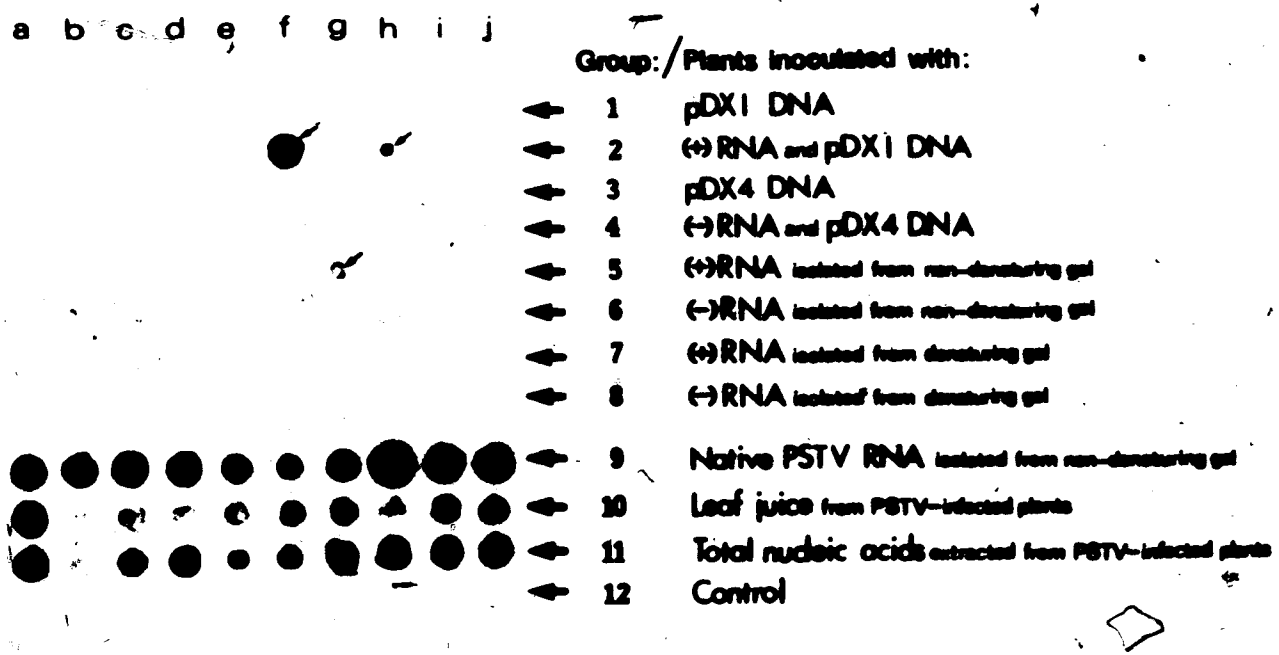
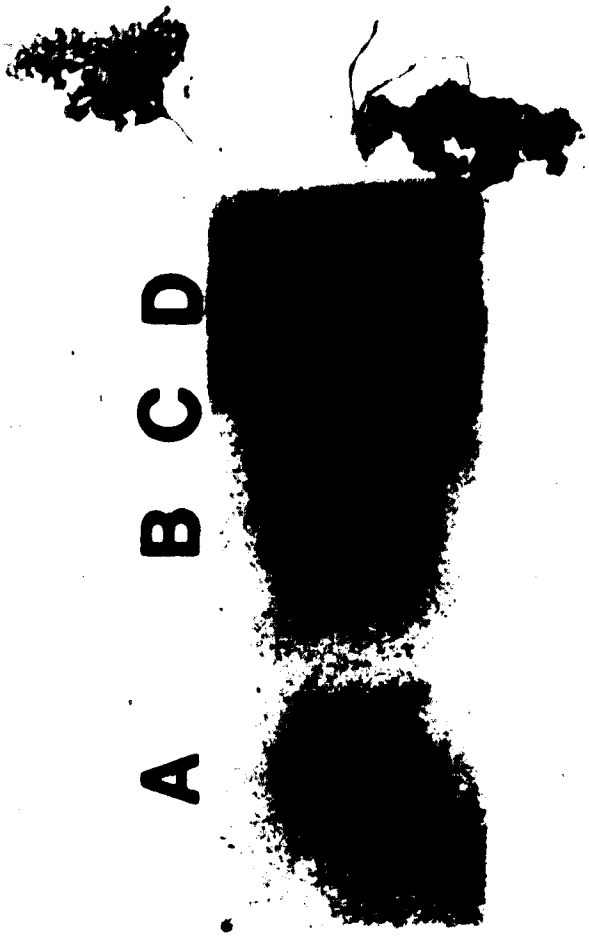

a b c d e f g h i j
Group:/Plants inoculated with:
1 pDX1 DNA
2 (+)RNA and pDX1 DNA
3 pDX4 DNA
4 (+)RNA and pDX4 DNA
5 (+)RNA isolated from non-denaturing gel
6 (-)RNA isolated from non-denaturing gel
7 (+)RNA isolated from denaturing gel
8 (-)RNA isolated from denaturing gel
9 Native PSTV RNA isolated from non-denaturing gel
10 Leaf juice from PSTV-infected plants
11 Total nucleic acids extracted from PSTV-infected plants
12 Control

Fig. 14. Spot hybridization for the infected plants using different probes. (a) Hybridization using nick translated PSTV cDNA as a hybridization probe. (b) Hybridization using nick translated pSP64 plasmid DNA as a hybridization probe. A: a plant infected by (+) RNA (group 5). B, C and D: plants infected by (+) RNA and pDXT DNA (group 2). Sample B: leaf juice from an infected plant developing the disease symptoms at 3 weeks. Samples C and D: leaf juice from infected plants developing the disease symptoms at 6 weeks.



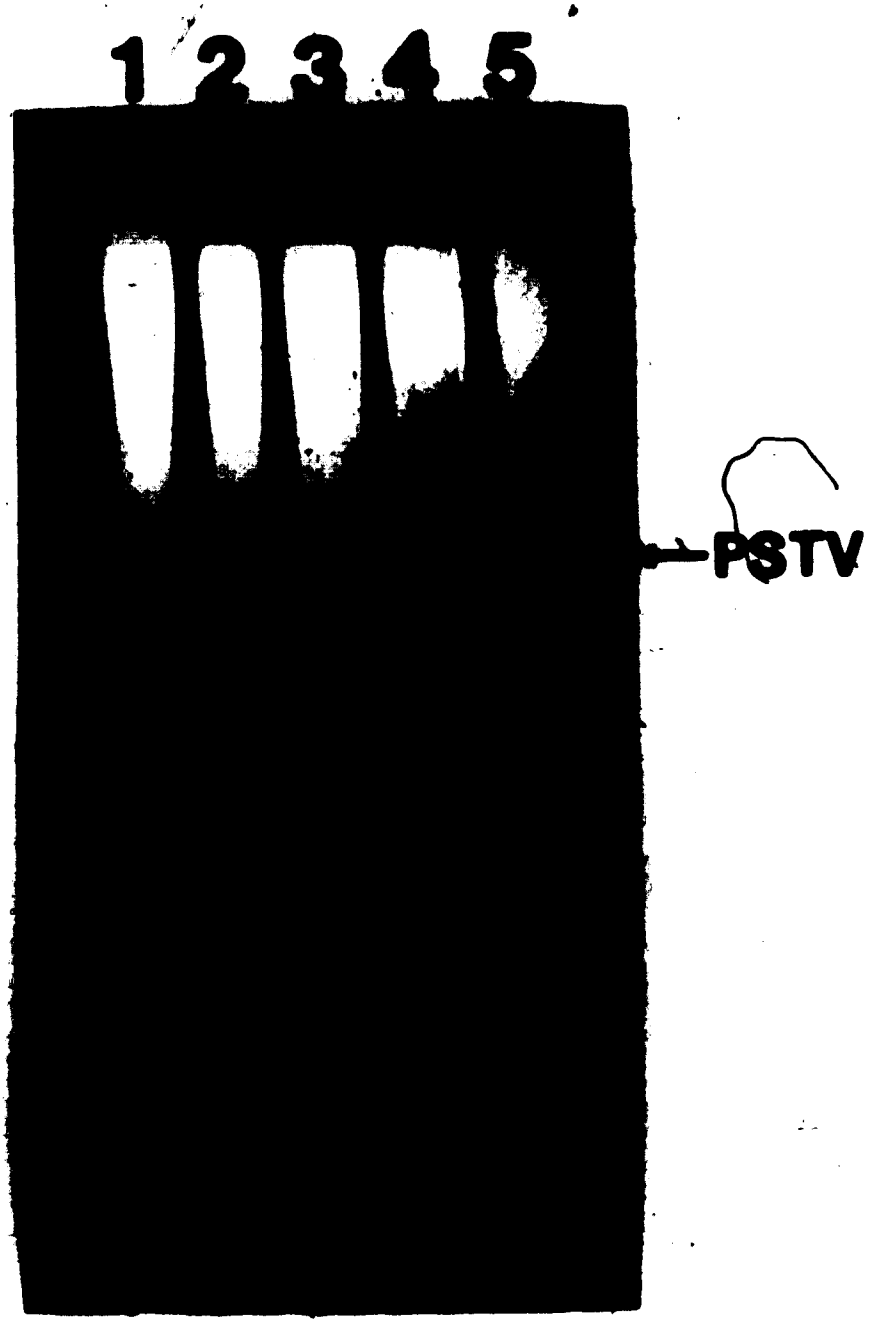
(a)

(b)

3.7 Characterization of PSTV RNAs extracted from (+) RNA as well as (+) RNA and pDX1 DNA inoculated tomato plants

To further examine the absence of the pSP64 and pSP65 vector derived polylinker sequences, the total nucleic acids from the diseased plants were extracted and examined by polyacrylamide gel electrophoresis. The results are shown in Fig. 15. In the earlier experiment (Fig. 12), a significant difference in size between the native PSTV RNA and the transcribed RNA was observed because the transcripts carried the extra polylinker sequences from the vector. In the results shown in Fig. 15, however, all the recovered RNAs from the experimental group (lane 2 to 5) were the same size as the native PSTV RNA (lane 1). These results further support the suggestion that the extra sequences were removed from the RNA transcripts during replication. Although the mechanism of this *in vivo* excision and religation reaction is not understood, this result is in good agreement with results of previous investigations from other laboratories (Tabler and Sanger, 1984; Robertson *et al.*, 1985; Hammond *et al.*, 1985).

Fig. 15. Detection of PSTV RNA by electrophoresis in a 5% polyacrylamide gel containing 8M urea. Lane 1: nucleic acids from a plant inoculated with native PSTV RNA. Lane 2: nucleic acids from a plant inoculated with (+) RNA transcripts from pDX1. Lanes 3 to 5: nucleic acids from three plants inoculated with a mixture of (+) RNA transcripts and pDX1 DNA, respectively.


1 2 3 4 5
PSTV

3.8 Characterization of the progeny RNA

In the above experiments, the disease symptoms in plants infected with control native PSTV were visible at two weeks after inoculation. In the experimental groups, one plant inoculated with (±) RNA transcripts and pDX1 DNA (group 2) exhibited the disease symptoms at 3 weeks after inoculation. Two additional plants from the same group and one plant in group 5, i.e. infected with (+) RNA transcripts isolated from a non-denaturing gel, exhibited the disease symptoms at 6 weeks after inoculation. In order to establish whether or not this observed delay in appearance of the disease symptoms is an intrinsic property of the viroid RNA recovered from the plants in different experimental groups, the following experiment was performed.

Total nucleic acid preparations extracted from the above mentioned four groups of the diseased plants served as a source of inoculum. Ten inoculated plants were used for each nucleic acid preparation. Typical viroid disease symptoms occured in all of the experimental plants at the same time as in the control plants inoculated with native PSTV RNA. Because a lower incubation temperature was used (22°C instead of 27°C), the symptoms were readily visible at 4 weeks after inoculation. This result suggests that the progeny RNAs recovered from the initial experiment exhibited the same level of virulence as the native PSTV.

4. Discussion

In this study, monomeric PSTV (+) and (-) RNA transcripts were obtained *in vitro* using the SP6 transcription system. The infectivity of these RNA transcripts was assayed by inoculating them onto indicator tomato plants and the properties of the progeny RNAs were characterized. The experimental program of this study can be summarized as follows.

(1) Subcloning the PSTV cDNA into pSP6 vectors and the transcription of (+) and (-) RNA transcripts *in vitro*

The plasmid pAV401 containing a full length PSTV cDNA was originally obtained from Dr. Pieter Vos (van Wezenbeck *et al.*, 1982). The cDNA insert was excised by digestion with BamH1 enzyme and examined by electrophoresis in a 5% polyacrylamide gel (Fig. 4). The size of the cDNA insert was confirmed by gel electrophoresis to be about 359 base pairs long. The insert cDNA was able to hybridize with nucleic acid preparations from PSTV diseased plants, but not with those from healthy tomato plants (Fig. 5). These studies indicate that the plasmid pAV401 does contain a full length PSTV cDNA copy.

The PSTV cDNA was then cloned into the BamH1 site of a transcription vector, pSP64. The cDNA was also used as a molecular probe to screen the transformed bacterial colonies for the presence of plasmids containing PSTV inserts. Eight bacterial colonies exhibiting positive hybridization signals were selected for further characterization. However, upon

digestion with SmaI to release the cDNA insert, only one out of the eight clones released a single DNA species with the expected size of PSTV cDNA as determined by gel electrophoresis. The rest of the colonies exhibited either a weakly staining DNA band or additional bands representing unknown DNA fragments upon gel electrophoresis. The recombinant plasmid selected in this experiment was later designated as pDX1. A computer analysis of PSTV cDNA (Fig. 3), predicted the presence of one SmaI restriction endonuclease site located near the BamH1 site in the viroid cDNA. This information was utilized to determine the orientation of the cDNA insert in pDX1 DNA. Since the expected RNA transcripts will have the same polarity and sequence as the viroid RNA, the cDNA insert on pDX1 was designated as the (+) orientation. In order to obtain the cDNA insert of the opposite orientation in the transcription vector, I took the advantage of the restriction endonuclease sites available in the polylinker sequence of the pSP64 and pSP65 vectors. A double digestion of the pDX1 DNA by HindIII and EcoRI restriction endonucleases released the PSTV cDNA flanked at both ends by short polylinker sequences. This DNA fragment, upon subcloning into the pSP65 vector, then yielded the plasmid bearing PSTV cDNA in the opposite orientation. It was expected that RNA transcripts of (-) polarity would be transcribed from this plasmid, which was designated as pDX4 (Fig. 10). The nature of the insert was confirmed by dot-blot hybridization using PSTV cDNA as the

hybridization probe (Fig. 8). Again, the orientation of the insert was confirmed by determination of the unique SmaI site (data not shown).

As shown in Fig. 11 and 12, RNA transcripts can indeed be generated from pDX1 and pDX4 DNA upon the addition of SP6 RNA polymerase. The run-off transcripts were of the expected sizes as shown by electrophoresis in 5% or 6% polyacrylamide gels. Transcription should originate at the RNA initiation site (position 1) of the SP6 promoter, continue through part of the polylinker sequence, through the PSTV cDNA, and through the rest of the polylinker sequence before terminating. The DNA terminus was generated by digestion with restriction endonucleases EcoRI (pDX1) or HindIII (pDX4). The additional sequences which were expected to be present on the (+) and (-) RNA transcripts were as follows:

(+) RNA transcript:

5'-GAATACAAGCTTGGGCTGCAGGTCGACTCTAGAG-----PSTV

RNA------CTTAAGCTCGAGCGGGCCCCTAG-3'

(-) RNA transcript:

5'-GAATACACGGAATTCGAGCTCGCCCGGG-----PSTV

RNA------TTCGAACCCGACGTCCAGCTGAGATCTCCTAG-3'

In this study, *in vitro* transcription of PSTV (+) and (-) RNA transcripts from pDX1 and pDX4 was efficient. In a one hour reaction, 6-10 μg of RNA transcripts were obtained from 1 μg of DNA template. Moreover, the run-off transcripts obtained in this system were quite uniform in size. A minor degree of heterogenity was observed in the size of (-) RNA

transcripts from pDX4 DNA (Fig. 12). Assuming that all the RNAs initiate at the predicted initiation site, the results suggest that premature termination occurred. Presumably the DNA sequence upstream from the HindIII site of the pDX4 vector is such that premature termination of transcription by the SP6 RNA polymerase might occur. On the other hand, the problem of snapback transcription associated with SP6 RNA polymerase (Melton *et al.*, 1984) was not observed.

(2) Infectivity of PSTV monomeric transcripts

The infectivities of the (+) and (-) RNA transcripts were examined by inoculation onto indicator tomato plants. The validity of the assay was established by the development of the viroid disease symptoms at 2 weeks after inoculation in all of the control plants inoculated with 3 different types of preparations of PSTV. None of the mock inoculated control plants developed the disease symptoms throughout the duration of the experiments (Table 1).

Three out of 10 plants developed the disease symptoms in group 2 where the plants were inoculated with (+) RNA transcripts mixed with pDX1 template DNA. Apparently the infectivity was derived from the RNA transcripts rather than from the DNA template since no infectivity was observed when the plants were inoculated with pDX1 DNA alone (group 1). Moreover, the RNA transcripts purified from non-denaturing gels were still able to infect at least 1 out of 10 plants (group 6). The reduced level of infectivity could be explained by possible degradation or alteration of the RNA

transcripts during the gel electrophoresis and subsequent extraction procedures. This interpretation was further confirmed by the lack of infectivity observed in the (+) RNA transcripts purified by electrophoresis on denaturing gels. Presumably contaminating urea and other components might further inactivate the RNA transcripts. Similar lack of infectivity of linear PSTV RNA isolated from denaturing gels was also observed by Morris (1979).

The delayed appearance of the disease symptoms was observed in the 3 plants inoculated with (+) RNA transcripts mixed with pDX1 DNA (group 2). One plant developed the symptoms beginning at 3 weeks after inoculation, whereas the other two developed the symptoms another 3 weeks later. When the leaf juice samples collected at 4 weeks after inoculation were examined by spot hybridization for the presence of PSTV RNA, the plants with the symptoms at 3 weeks gave a strongly positive hybridization signal, as did all the 30 plants inoculated with control PSTV RNA. Leaf juice from one of the plants with symptoms developed at 6 weeks showed a weaker but definitely positive hybridization signal, whereas the other one exhibited a negative signal. Therefore, the concentration of intracellular viroid RNA correlates well with the appearance of the disease symptoms. Similarly, a weakly positive hybridization signal was observed for the single plant inoculated with (+) RNA transcripts in group 5. Leaf juice from these 4 diseased plants taken at 6 weeks after inoculation gave positive

hybridization signals with the PSTV cDNA probe (Fig. 14). These results also support the above interpretation for the correlation between the disease symptoms and amount of intracellular viroid RNA.

The progeny RNAs harvested from these 4 groups of the diseased plants were inoculated onto indicator tomato plants for further characterization. It was shown that they were all able to induce the viroid disease symptoms and also that the kinetics of the appearance of the symptoms was indistinguishable from those produced by inoculation of native PSTV RNA. Moreover, the progeny RNAs exhibited the same electrophoretic mobility as the native PSTV RNA. As discussed above, the progeny RNAs were able to hybridize with a PSTV cDNA probe in a spot assay, but not with the parental plasmid pSP64 bearing the polylinker sequence. Taken together, these results suggest that the progeny RNA lacks the extra polylinker sequences present on the RNA transcripts in the inoculum. Apparently, the polylinker sequences were excised from the RNA transcripts *in vivo*. The linear PSTV sequences were religated into the circular viroid structure before efficient RNA replication occurred. The delay in the appearance of the disease symptoms as well as the delayed accumulation of viroid RNA (in plants in groups 2 and 5) can be interpreted as the latent period required for the excision of the polylinker sequences and religation of viroid RNA. Presumably, the lack of infectivity in the other samples in the same experimental

groups was probably due to difficulty encountered in excision and/or religation to form a replicating PSTV RNA molecule.

The recovery of progeny RNA apparently identical to the PSTV RNA, rather than PSTV bearing extra sequences, was also observed by Robertson *et al.* (1985), Tabler and Sanger (1985), and Hammond *et al.* (1985). Since the extra polylinker sequences were present in the core region of the PSTV RNA as proposed by Keese and Symons (1985), these results further support the suggestion that this region has a highly conserved sequence and its integrity is essential for viroid replication.

Furthermore, the generation of progeny RNA apparently identical to the native PSTV RNA would suggest the presence of enzyme systems capable of specific recognition, excision of the extra sequences and religation into PSTV viroid RNA. In fact, an RNA ligase was found in wheat germ extracts which can efficiently circularize native linear PSTV RNA *in vitro* (Konarska *et al.*, 1981; Branch *et al.*, 1982; Kikuchi *et al.*, 1982).

Previously, Cress *et al.* (1983) inserted the PSTV cDNA monomer downstream from the Lac promoter and induced its transcription *in vivo*. The (+) RNA transcripts of undefined size and low infectivity were generated. However, multimeric RNA transcripts of (+) polarity were showed to be infectious when inoculated onto indicator tomato plants (Robertson *et al.*, 1985; Tabler and Sanger, 1985). The present study

presents the evidence that monomeric (+) RNA transcripts of defined size obtained from the SP6 transcription system *in vitro* could show infectivity.

(3) Lack of infectivity in the (-) RNA transcripts and relevance of this observation to the replication of viroid RNA

As shown in Table 1, none of the (-) RNA transcripts in groups 4, 6 and 8 exhibited infectivity when inoculated onto indicator tomato plants. None of the above groups gave positive hybridization signals for viroid RNA with the PSTV cDNA probe in the dot-blot hybridization assay as shown in Fig. 13. These results indicate that monomeric (-) RNA transcripts are not able to initiate RNA replication *in vivo*. The block in RNA replication could be in the excision of extra sequences, religation or in some other steps. The previous findings that multimeric (-) RNAs were non-infectious (Tabler and Sanger, 1985) is consistent with this observation.

The above observation has special relevance to the current knowledge on the replication of viroid RNA. Branch and Robertson (1984) proposed two alternate schemes for the replication of viroid RNA. In scheme A, (+) RNA is transcribed into a multimeric (-) RNA, which in turn serves as a template for the transcription of a multimeric (+) RNA. Each monomer is excised and religated into a (+) circular monomeric RNA. In scheme B, (+) RNA is transcribed into a multimeric (-) RNA which is then cleaved into monomeric (-)

RNAs. The monomeric (-) RNA served as a template for transcription of a multimeric (+) RNA which is cleaved to form monomeric (+) RNAs. One of the key features which would enable one to distinguish between these two alternate schemes is the presence of monomeric (-) RNA in scheme B. The lack of infectivity of the (-) monomeric RNA observed in this study would support scheme A, rather than scheme B. In the light of the non-infectivity of multimer and monomer (-) RNAs, it appears that, unlike multimer (+) RNA of PSTV, multimer (-) RNA of PSTV is present in the replication cycle only as a replicational intermediate and a template for synthesizing multimer (+) RNA, but not for generating monomer (-) RNA. In fact, circular (-) RNA molecules have never been found in PSTV infected cells.

Information obtained in studies on PSTV replication done by using an *in vitro* transcription system will facilitate the study of the relationship of viroid structures and functions. Mutations in viroid cDNA can be constructed at the DNA level. The *in vitro* transcription system would allow synthesis of corresponding mutant RNA molecules for analysis of viroid infectivity and virulence. This would permit futher investigation on the replication and pathogenicity of viroid RNA molecules.

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