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Infectivity assays of in vitro and in vivo transcripts of papaya ringspot potyvirus

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Abstract. A full-length cDNA with nucleotide sequence representing the genomic RNA of a Hawaii strain of papaya ringspot potyvirus (PRSV HA) was constructed downstream from a bacteriophage T3 promoter in an in vitro transcription vector. The plasmid was able to generate an in vitro transcript corresponding to PRSV RNA (10326 nt) with one extra guanosine residue at the 5' terminus and 12 nonviral nucleotides at the 3' end following a poly(A)36 tract. In vitro translation products and immunoprecipitation analysis with the antiserum to PRSV verified correctness of the gene expression of the transcript. When the capped transcript was mechanically introduced to the systemic host papaya and the local lesion host *Chenopodium quinoa*, typical symptoms of PRSV HA appeared at almost the same time as on those host plants inoculated with native PRSV RNA. Western blotting and serologically specific electron microscopy with PRSV antiserum confirmed the infection. The uncapped in vitro transcript and the transcript with longer nonviral nucleotides (64 nt) at the 3' end were not infectious. The full-length cDNA was also constructed with a cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase (NOS) terminator in an in vivo expression vector. Purified plasmids were applied directly onto host plants either mechanically or by bombardment with a particle delivery system to analyze their infectivity. The plasmid without extra nucleotides between the 35S promoter and the 5' end of PRSV sequence and with 10 nonviral residues and a *Not*I site at its 3' end was infectious, as evident from symptom development, and ELISA, immunodiffusion, and serologically specific electron microscopy analyses with PRSV antiserum. The construct with 33 nonviral nucleotides at the 5' end of the PRSV sequence and more than 64 nucleotides at the 3' end was not infectious. The infectious in vitro and in vivo transcripts derived from the full-length cDNA to PRSV RNA are the longest so far recorded for a plant virus.

Keywords: In vitro transcript; In vivo transcript; Papaya ringspot potyvirus.

Introduction

Papaya ringspot virus (PRSV) is a member of the genus *Potyvirus*, the largest group among plant viruses (Ward and Shukla, 1991), and is the major limiting factor for papaya production throughout tropical and subtropical areas (Purcifull et al., 1984). PRSV particles are flexuous rods, 700–800 nm in length, and contain a monopartite genome consisting of a positive-sense ssRNA with a 5' genome-linked protein (VPg) (Siaw et al., 1985; Riechmann et al., 1989; Murphy et al., 1990) and a 3' poly(A) tract (Hari et al., 1979).

The complete nucleotide sequences of a severe Hawaii strain PRSV HA and a mosaic Taiwan strain PRSV YK have been determined (Yeh et al., 1992; Wang, 1993). The PRSV genome contains 10326 nucleotides excluding the 3' poly(A) tail and is the longest encountered among the sequenced potyviruses. PRSV RNA encodes a large polyprotein of 381 kDa (Yeh and Gonsalves, 1985; Yeh et al., 1992) which is processed into 8 or 9 final products via three virus-encoded proteinases (P1, HC-Pro, and NIa). Like other potyviruses, the proposed genetic organization of PRSV RNA is VPg - 5' leader - P1 (63K) - HC Pro - P3 (46K) - CI - P5 (6K) - NIa - NIb - CP - 3' noncoding region - poly(A) tract (Yeh et al., 1992). The P1 protein of PRSV is the most variable protein among potyviruses, and it has an M_r of 63 kDa, which is 18–34 kDa larger than those of the other potyviruses (Yeh et al., 1992).

Infectious transcripts constructed from cDNA clones of plant and animal RNA viruses have been well documented (Boyer and Haenni, 1994). In plant viruses, infectious in vitro RNA transcripts were first successfully prepared for brome mosaic virus (BMV) (Ahlquist and Janda, 1984), and the construction of a directly infectious cDNA clone was first reported for RNA 3 of alfalfa mosaic virus (AlMV) through coinoculation with RNAs 1, 2, and 4 (Dore and Pinck, 1988). In the potyvirus group, infectious in vitro transcripts have been synthesized from full-length cDNA clones with bacterial phage promoters for tobacco vein mottling virus (TVMV) (Domier et al., 1989), plum pox virus (PPV) (Riechmann et al., 1990), zucchini yellow mosaic virus (ZYMV) (Gal-On et al., 1991), and tobacco etch virus (TEV) (Dolja et al., 1992). In vivo infectious clones have been obtained for PPV (Maiss et al., 1992), ZYMV (Gal-On et al., 1995) and potato virus Y (PVY) (Fakhfakh et al., 1996), which were driven by a cauliflower mosaic virus (CaMV) 35S promoter and ter-

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minated by NOS terminator. The ability to generate biologically functional RNA transcripts from full-length cDNA clones, either by in vitro transcription or by direct in vivo transcription, has provided a powerful tool to study gene functions, virus replication, and pathogenicity by site-specific mutation and recombinant DNA techniques.

In this investigation, the full-length cDNA clone of a severe Hawaii strain PRSV HA was constructed either with the bacteriophage T3 promoter for generating an in vitro transcript or with the CaMV 35S promoter for an infectious plasmid. The biological activities were analyzed on the systemic host *Carica papaya* L. and the local lesion host *Chenopodium quinoa* Willd. by mechanical inoculation or particle bombardment. The 10.3 kb constructs of PRSV full-length cDNA generated the longest infectious in vitro and in vivo transcripts among the positive-sense monopartite plant viruses so far recorded.

Materials and Methods

Construction of a Full-Length cDNA Clone of PRSV RNA with a T3 Promoter

PRSV HA, a severe strain originated from Hawaii (Yeh et al., 1984), was propagated in plants of *Cucumis metuliferus* (Naud.) Mey. (Provvidenti and Gonsalves, 1982). Virus purification, viral RNA preparation, cDNA synthesis, and cloning strategies were previously described (Yeh et al., 1992). The strategy for the construction of full-length cDNA clones pT3-Xb and pT3-HAG of PRSV RNA is summarized in Figure 1. Four overlapping cDNA clones, including pHA10, λgt11-AI, λgt11-CI, and pHA11, were used for construction of full-length cDNA clones to PRSV HA RNA. Clone λgt11-AI with an insert of 4.9 kb was immunoscreened from a λgt11 cDNA library (Yeh et al., 1992) by reactions with both of the antisera to amor-

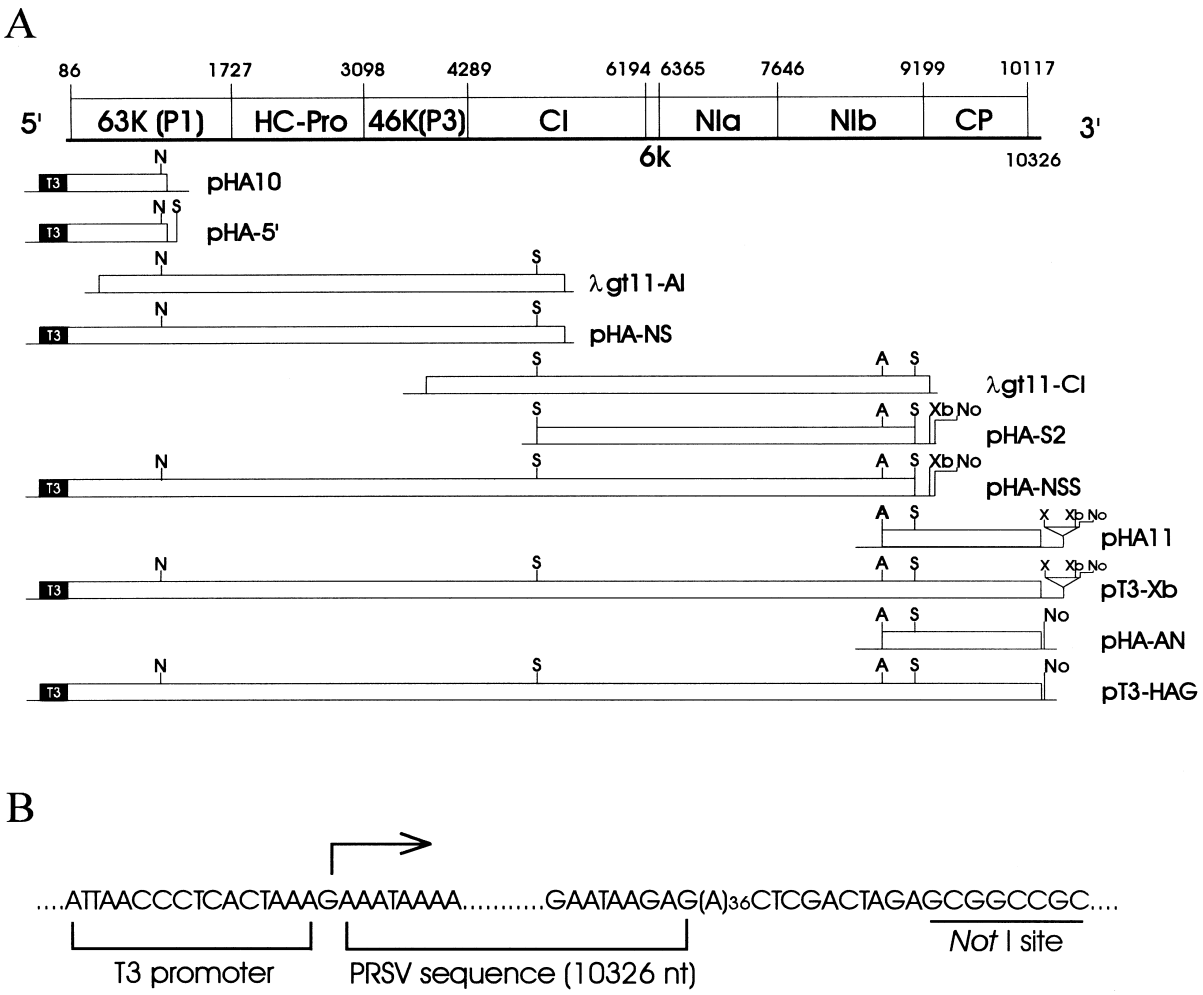

Figure 1. A, construction of full-length cDNA clones (pT3-Xb and pT3-HAG) of PRSV RNA downstream from a T3 promoter. The full-length cDNA clones were constructed by ligating suitable restriction fragments from four overlapping cDNA clones corresponding to different genomic regions of PRSV HA (a Hawaii strain). Restriction sites relevant for the construction are shown: N, *Nhe*I; S, *Spe*I; A, *Apa*I; X, *Xho*I; Xb, *Xba*I; No, *Not*I. B, predicted site of initiation and direction of transcription initiated by T3 RNA polymerase is indicated by an arrow. The junction between the T3 promoter and the 5' end of PRSV sequence (10326 nucleotides) contained an extra guanosine residue, and the 3' end of the PRSV sequence was followed by a poly(A)36 tract and 10 nonviral residues before the *Not*I site.

phous inclusion protein (AIP) (Yeh and Gonsalves, 1985) and to cylindrical inclusion protein (CIP) (Yeh and Gonsalves, 1984) of PRSV. Clone λgt11-CI with an insert of 5.5 kb reacted with both of the antisera to PRSV CIP and to CP (Yeh et al., 1984) was also selected from the same library. Clone pHA11 with an insert of 1.2 kb reacted with the antiserum to CP (Yeh et al., 1984) was selected from a λZAP II cDNA library (Yeh et al., 1992). Clone pHA10 representing the 1 kb region of the 5' end of PRSV RNA was obtained by cloning the cDNA fragment amplified by reverse transcription-polymerase chain reaction (RT-PCR) with primers specific to nucleotide positions 1 to 24 and 1044 to 1065 of PRSV RNA (Sambrook et al., 1989; Yeh et al., 1992).

The selected four cDNA clones covering the complete genomic PRSV RNA were used to construct the full-length cDNA by ligating overlapping fragments that shared identical restriction sites. The polylinker sequence (49 nt) located between the T3 transcription initiation site and the 5' terminus of the PRSV sequence in pHA10 was removed by site-directed mutagenesis using an Amersham (Buckinghamshire, England HP7 9NA) Sculptop in vitro mutagenesis kit. *Escherichia coli* strain XL-1 was used for the cloning of all plasmids and the production of single-stranded DNA via helper phage VCSM13 (Stratagene, La Jolla, California) for the mutagenesis reaction. The oligonucleotide used in the reaction, 5'-ACCCTCACTAAAG AAATAAAACATCTC-3', contained 13 nucleotides of the T3 promoter with an additional guanosine residue, which was followed by the first 14 nucleotides of the 5' end of PRSV sequence. The resulting plasmid pHA-5' was sequenced to verify the deletion.

Plasmid pHA-5' was digested with *Nhe*I and *Spe*I and ligated with *Nhe*I-*Spe*I digested fragment (4212 bp) liberated from λgt11-AI clone to generate pHA-NS. This plasmid was then digested with *Spe*I and ligated with a *Spe*I fragment (4020 bp) liberated from pHA-S2, which contained a *Spe*I fragment from clone λgt11-CI. The resulting plasmid pHA-NSS contained a cDNA insert spanning PRSV genomic residues 1 to 9182. Finally, the full-length cDNA clone of PRSV, pT3-Xb, was obtained by ligating *Apa*I-*Xba*I digested pHA-NSS with a *Apa*I-*Xba*I fragment (1369 bp) from pHA11 (Figure 1A).

Another full-length cDNA clone of pT3-HAG was constructed just as pT3-Xb was, except for the last step. The cDNA insert of pHA11 was shortened by digestion with *Xho*I and *Xba*I to generate pHA-AN, in which most of the nonviral nucleotides between the poly(A) tail and the *Not*I site were removed. The *Apa*I-*Not*I insert (1317 bp) from pHA-AN was subcloned into the same restriction sites of pHA-NSS to generate the full-length cDNA clone pT3-HAG (Figure 1A). Both pT3-Xb and pT3-HAG were used for in vitro transcription and infectivity assay.

Construction of a Full-Length cDNA Clone of PRSV RNA with a 35S Promoter

A plasmid pCaMVCN containing a CaMV 35S RNA promoter and a NOS terminator was purchased from Pharmacia/LKB (South Plainfield, NJ 07080). *Escherichia coli* strain JM109 was used for the cloning of the plasmid, and *E. coli* strain XL-I was used for site-directed mutagenesis experiments.

The strategy used for the construction of a full-length cDNA clone of PRSV with the 35S promoter is outlined in Figure 2. The *Hin*dIII-*Sac*I fragment of pHA10 was filled-in with the Klenow fragment of DNA polymerase I, and ligated to pCaMVCN that was digested with *Sal*I and filled-in by the Klenow fragment. The resulting plasmid 35S-5' contained 33 extra nucleotides between the 35S promoter and the 5' end of PRSV sequence. The full-length cDNA clone of PRSV, p35S-Xb, was then obtained by ligating *Nhe*I-*Not*I fragment (9476 bp) liberated from pT3-Xb into the same restriction sites of p35S-5'.

Another full-length cDNA clone p35S-HA containing the 35S promoter was constructed as follows. In order to generate single-strand DNA for site-directed mutagenesis, p35S-5' was further digested with *Nar*I and *Cla*I and filled-in with the Klenow fragment and subcloned into *Pvu*II-digested pBluescript II (KS+) (Stratagene) to create p35S-5'KS. The 33 nonviral nucleotides located between the transcription initiation site of the 35S promoter and the first nucleotide of PRSV sequence in p35S-5'KS were then removed by site-directed mutagenesis. The oligonucleotides used in the reaction, 5'-TTTATTTCCTCTCC AAATGAAATGAA-3', contained the first 7 nucleotides of PRSV RNA followed by the last 19 nucleotides of the 35S promoter. The resulting plasmid p35S-5'mut was sequenced to verify the deletion.

Plasmid p35S-5'mut was further digested with *Xba*I and then cloned back into pCaMVCN at the same enzyme sites to generate p35S-5'CN. This plasmid was then digested with *Nco*I and ligated to a *Nco*I fragment (4494 bp) liberated from the full-length cDNA clone of pT3-HAG to produce p35S-NCO. Finally, a *Mlu*I-*Not*I fragment (7113 bp) excised from the cDNA clone pT3-HAG was ligated to *Mlu*I-*Not*I digested p35S-NCO to generate p35S-HA (Figure 2). Both p35S-Xb and p35S-HA were directly used for infectivity assay.

Synthesis of In Vitro Transcripts from the Full-Length cDNA Clones of PRSV RNA with a T3 Promoter

In vitro transcription of *Xba*I-linearized pT3-Xb or *Not*I-linearized pT3-HAG (30 µg in 200 µl final volume) was carried out with the mCAP mRNA capping kit (Stratagene), T3 RNA polymerase (1.2 unit per µl) (Pharmacia), RNase inhibitor (Promega, Madison, WI 53711-5399) (0.4 unit per µl), and 500 mM m^7GpppG (New England Biolabs, Beverly, MA 01915). The mixture was first incubated at 37°C for 1 h, GTP was then supplemented to 250 mM and the incubation was further proceeded for 1 h. Size and concentrations of the transcripts were estimated by comparison with positions and band intensity of a known quantity of viral RNA.

To test the messenger activity of the in vitro transcripts, the synthesized transcripts and viral RNA of PRSV were

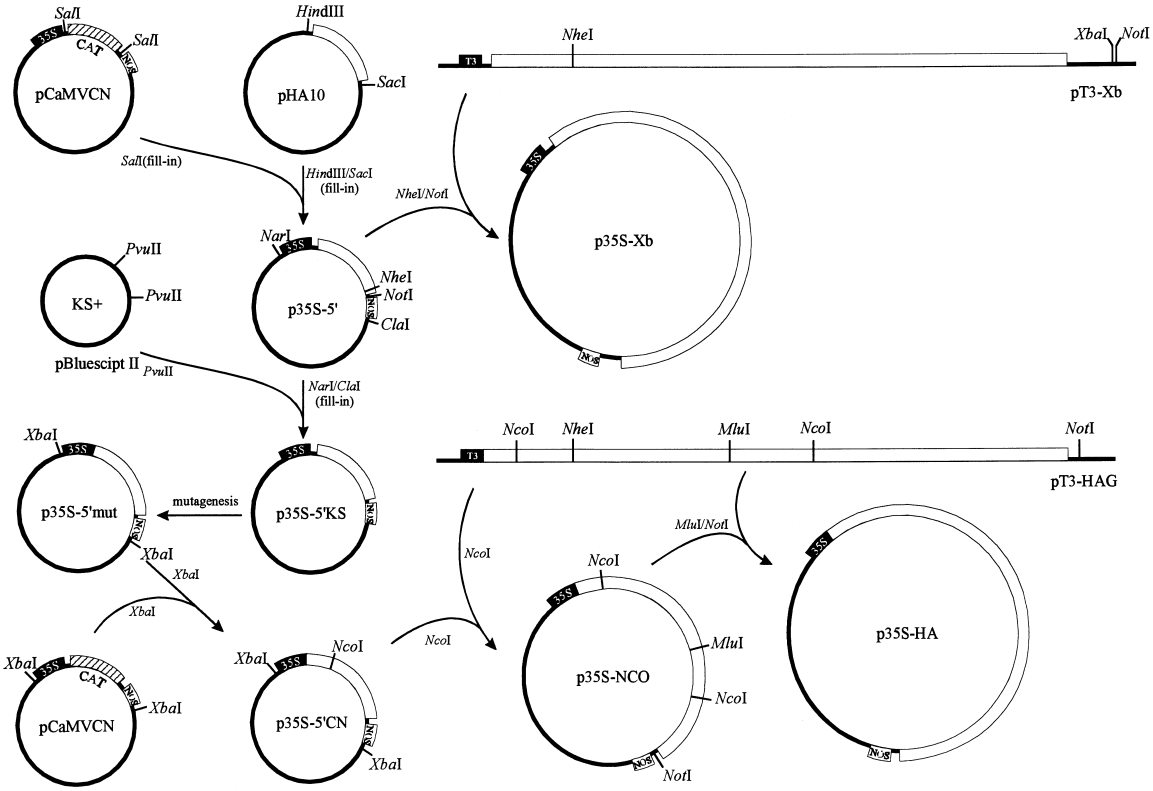

Figure 2. Construction of plasmids containing the full-length PRSV cDNA with a 35S promoter and a NOS terminator. The clones and steps employed to generate p35S-Xb and p35S-HA are schematically presented. The p35S-Xb contained 33 nonviral nucleotides between the 35S promoter and the 5' end of the PRSV sequence and 64 extra nonviral residues before a *Xba*I site at the 3' end of the PRSV sequence following the poly(A) tail. The nonviral nucleotides in between the 35S promoter and the 5' end of the PRSV sequence was completely removed in p35S-HA, and the nonviral sequence upstream of the *Not*I site was trimmed to 10 nucleotides. Both plasmids contained a NOS terminator.

used for in vitro translation and immunoprecipitation analyses. In vitro translation was carried out in the rabbit reticulocyte lysate system (Stratagene) using [^{35}S] methionine (1000 Ci/mmol) for labeling products. Immunoprecipitation with the antiserum to PRSV (Yeh et al., 1984) followed the procedure described by Dougherty and Hiebert (1980).

Plant Inoculations

Plants used for inoculation included the systemic host *Carica papaya* L. at the 2–3 true-leaf stage and the local lesion host *Chenopodium quinoa* Willd. with four fully expanded leaves. In vitro transcripts from linearized pT3-Xb and pT3-HAG without further treatment were used for infectivity assays. Transcription mixtures of 20 µl (final quantity of about 2–3 µg RNA) were mechanically applied onto a leaf of each carborundum-dusted plant with a sterilized glass spatula. Healthy seedlings were mock-inoculated with 20 µl transcription buffer or with 0.5 µg of PRSV native RNA per plant as controls.

PRSV full-length cDNA clones p35S-Xb and p35S-HA were purified from cesium chloride gradients (Sambrook et al., 1989). Aliquots of 20 µl, containing 15–20 µg DNA in sterilized water, were mechanically applied onto carborundum-dusted plants with a sterilized glass spatula. Plants were also inoculated by microprojectile bombardment with a particle delivery system (Bio-Rad, Biolistic PDS-1000/He, Hercules, California 94547) following the procedure described in the user's manual. Briefly, aliquots of 10 µl (containing about 0.8 µg DNA) of the DNA-coated gold microcarriers (1.6 µm) were transferred onto the center of the macrocarrier and dried in low humidity under vibration-free conditions. Fifteen papaya seedlings at the stage of one true leaf were clustered by rubber bands and placed on a petri dish with the leaves facing the macrocarrier. The petri dish was placed on the holder in the third position down from the microcarrier launch assembly. The vacuum was maintained at the desired vacuum level of 20–25 inches of mercury, and then the microcarriers were delivered into the plant tissue with pressured helium gas which ruptured the disks at 650 psi. All inoculated plants were kept in a temperature-controlled (23–28°C) greenhouse for observation of symptom development.

Confirmation of Infection

Inoculated plants were assayed by ELISA using PRSV antiserum (Yeh et al., 1984) to detect the presence of PRSV 2–3 wk after inoculation. Results were recorded by measurement of the absorbency at 405 nm by an ELISA reader (SLT-Labinstruments, Salzburg, Austria).

Total proteins extracted from leaf tissues of plants 3 wk after inoculation were separated by SDS-polyacrylamide gel electrophoresis, transblotted onto nitrocellulose membranes (Millipore Co.) and immunoanalyzed with the polyclonal antiserum to PRSV (Yeh et al., 1984) as described by Gooderham (1984).

SDS-immunodiffusion tests were conducted as described by Purcifull and Batchelor (Purcifull and Batchelor, 1977). Crude antigens were prepared from freshly harvested leaves of inoculated papaya plants. Undiluted antiserum to PRSV (Yeh et al., 1984) was added to appropriate wells, and the plates were incubated in a moist chamber at room temperature for 18–36 h.

Crude extracts from leaves of papaya plants with typical PRSV symptoms were mixed with an equal volume of 1:400 diluted PRSV antiserum (Yeh et al., 1984) and stained with aqueous uranyl acetate. Results of immunodecoration were examined with a JEOL 200 CX electron microscope and photographed with Kodak 4489 EM film.

Results

Full-Length cDNA Clones with a T3 Promoter or a 35S Promoter

The clone pT3-Xb contained the full-length cDNA sequence to PRSV RNA (10326 nt) downstream from the T3 transcription site, with one extra G upstream from the 5' end of the PRSV sequence and a poly(A) tail of 36 nucleotides at the 3' end followed by 64 nonviral nucleotides before a *Xba*I site for run-off transcription (Figure 1A). There is a risk with this construct that the 3'-nonviral extension may inhibit the infectivity. Therefore, another full-length cDNA clone was constructed by trimming nonviral nucleotides following the poly(A) tract. The resulting pT3-HAG clone was the same as pT3-Xb at the insert 5' end, but there were only 12 nonviral nucleotides at the 3' end after digestion with *Not*I for in vitro transcription (Figure 1B). Both of these two full-length cDNA clones were further used to construct full-length cDNA clones with a CaMV 35S promoter.

The plasmid p35S-Xb was constructed with 33 nonviral nucleotides between the 35S promoter and the 5' end of the PRSV sequence and contained a long polylinker sequence with 64 nucleotides before the *Xba*I site downstream from the poly(A) tract (Figure 2). This construct was used to investigate whether the nonviral nucleotides would influence the ability of infection. Construction of another full-length cDNA construct without any extra nucleotides downstream from the 35S promoter was more laborious than for p35S-Xb. Initially, the intention was to construct a full-length cDNA by ligating *Nhe*I-*Not*I digested p35S-5'mut with a *Nhe*I-*Not*I fragment (9388 bp) liberated from the cDNA pT3-HAG, but it was not successful. Therefore, the p35S-5'mut was subcloned back to pCaMVCN in *E. coli* strain JM109 to generate p35S-5'CN (Figure 2). Because it is easier to ligate a small fragment than a large one, a two-step ligation was performed to complete the full-length cDNA construct p35S-HA (Figure 2). This plasmid contained the first nucleotide of the PRSV sequence at the initiation site for transcription driven by the 35S promoter, the complete PRSV cDNA which carried a 36 poly(A) tract with 10 additional nucleotides and a *Not*I site, and a NOS polyadenylation signal as a terminator.

In Vitro Transcription, In Vitro Translation and Immunoprecipitation

The transcription products of pT3-Xb and pT3-HAG with or without cap structure were similar in size to native PRSV RNA extracted from virions (data not shown). The yields of the transcripts were about 0.8–1.0 µg from 1 µg of DNA.

The in vitro translation products generated by the capped or uncapped transcript of pT3-HAG were similar to those produced by authentic PRSV RNA (Figure 3 lanes 1, 2, and 3). The transcript of pT3-Xb also generated a similar pattern when it was translated in the same system (data not shown). The immunoprecipitation patterns of the translation products of the in vitro transcript of pT3-HAG and of the viral RNA were similar, and a 36 kDa protein corresponding to the coat protein of PRSV was detected in both cases (Figure 3 lanes 7–9). None of the translation products reacted with normal serum (Figure 3 lane 4–6). The immunoprecipitation result of the in vitro transcript of pT3-Xb also was similar to that of the in vitro transcript of pT3-HAG (data not shown). These results indicated that both full-length PRSV transcripts generated by the T3 promoter from pT3-HAG and pT3-Xb contained a correct open reading frame which was translated into normal viral products similar to those generated by the authentic PRSV RNA.

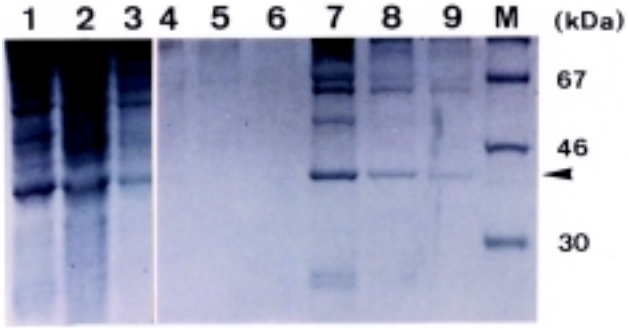

Figure 3. In vitro translation and immunoprecipitation analyses of the transcripts generated from the cloned full-length PRSV cDNA. Total in vitro translation products generated from PRSV native RNA (lane 1) were compared with those from in vitro capped and uncapped RNA transcripts generated from pT3-HAG (lanes 2 and 3, respectively). Immunoprecipitation analysis of translation products from in vitro transcripts was conducted with normal serum (lanes 4–6) and with the antiserum to PRSV (Yeh et al., 1984) (lanes 7–9). Transcripts used in lanes 2, 5, and 8 were capped, whereas those used in lanes 3, 6, and 9 were uncapped. Lane M, [^{14}C]-labeled protein markers. A 36 kDa protein corresponding to the coat protein of PRSV is indicated by an arrow head.

Infectivity Assay of In Vitro Transcripts

In two separate experiments, the capped in vitro transcript derived from pT3-HAG was shown to be infectious in the systemic host *C. papaya* by mechanical inoculation. Typical symptoms of mosaic and leaf distortion appeared on an average of 56% (49 out of 88 plants inoculated) of the seedlings 9–12 days after inoculation (Table 1), at about the same time as observed on those plants inoculated with native PRSV RNA. Papaya seedlings were also inoculated with the capped transcript derived from pT3-Xb or the uncapped transcript derived from pT3-HAG. The inoculated plants were maintained for the same period under the same conditions as above. None of these plants became infected while they were observed up to two months. All inoculated plants were also analyzed by ELISA with the antiserum to PRSV. Plants that developed visible symptoms produced strong positive readings, while the symptomless papaya showed negative reactions (data not shown). By immunoblotting analysis, a 36 kDa protein corresponding to the coat protein of PRSV was detected in the plants infected by the transcript of pT3-HAG (data not shown). By electron microscopy, numerous filamentous virus particles 750–800 nm in length were observed in the samples from transcript-infected plants. When the crude sap was treated with the antiserum to PRSV, the particles were specifically decorated (Figure 4).

Leaves of each test plant of the local lesion host *C. quinoa* were inoculated with 2–3 μg of the full-length transcript or 0.5 μg native viral RNA per leaf. Native PRSV RNA caused one to three times more lesions than those induced by the capped transcript derived from pT3-HAG (Table 1). No lesions were observed when the plants were inoculated with the uncapped RNA transcript from pT3-HAG or with the capped transcript from pT3-Xb. The results revealed that the capped transcript from pT3-HAG was infectious to both the systemic and local lesion host plants, whereas the capped transcript from pT3-Xb and the uncapped transcript from pT3-HAG were not infectious.

Infectivity Assay of Full-Length cDNA Clones with 35S Promoter

Plasmids containing the full-length PRSV cDNA with the 35S promoter were extracted from *E. coli* strain JM109

Table 1. Infectivity assay of in vitro transcripts derived from cloned PRSV full-length cDNA on the systemic host *Carica papaya* and local lesion host *Chenopodium quinoa*.

Inculum	*Carica papaya* [a]		*Chenopodium quinoa* [b]	
	No. of plants infected/ No. of plants tested	Infectivity (%)	No. of leaf inoculated	Infectivity (No. of lesions/leaf)
Mock inoculation with buffer	0/20	0	8	0
PRSV RNA	16/20	80	12	25
Capped transcript of pT3-Xb [c]	0/52	0	40	0
Capped transcript of pT3-HAG	49/88	56	12	15
Uncapped transcript of pT3-HAG	0/32	0	12	0

[a]Plants at the stage of 2–3 true leaves were inoculated with transcription buffer (mock), PRSV native RNA (0.5 μg per plant), capped or uncapped in vitro transcripts (2–3 μg per plant).

[b]Plants with 4 leaves were inoculated with transcription buffer (mock), PRSV native RNA (0.5 μg per leaf), capped or uncapped in vitro transcripts (2–3 μg per leaf).

[c]pT3-Xb was a PRSV full-length cDNA clone similar to pT3-HAG except that it contained additional 64 nonviral residues following the poly(A)36 track before the *Xba*I site.

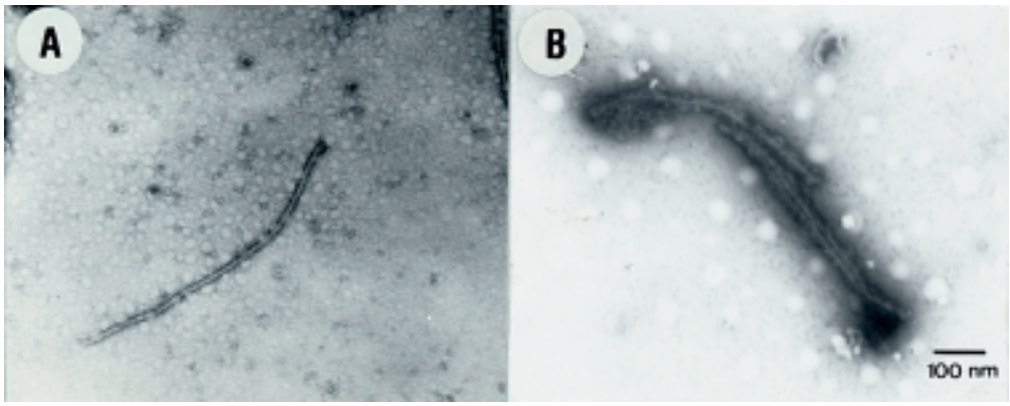

Figure 4. Serologically specific electron microscopy without (A) and with (B) the antiserum to PRSV performed on the leaf sap from a papaya plant infected with in vitro transcript of pT3-HAG. Filamentous particles 750–800 nm in length were observed (A), and the particles were strongly decorated by the antiserum (B).

with a final concentration of 1 µg/µl. A total of 355 papaya plants and 40 leaves of 10 *C. quinoa* plants were used for infectivity assay with the construct p35S-Xb. All plants inoculated mechanically or by particle-bombardment with DNA from p35S-Xb, up to 15–20 µg per plant, did not show infection in papaya or *C. quinoa*, as judging from negative ELISA reactions (data not shown) and the lack of symptom development (Table 2). However, when purified DNA from the clone p35S-HA was applied mechanically onto papaya plants, symptoms were observed, 8 to 9 days after inoculation, indistinguishable from those on plants mechanically inoculated with PRSV HA. Appearance of the symptoms induced by p35S-HA was not significantly delayed when compared with those induced by the virus. There were 24 out of 57 mechanically inoculated plants of *C. papaya* that developed symptoms (Table 2). Plants of *C. quinoa* developed symptoms 9 to 11 days after mechanical inoculation with p35S-HA. The local lesions were similar to those induced by PRSV.

When papaya plants were inoculated by microprojectile bombardment with p35S-HA-coated gold particles, typical symptoms of PRSV HA were observed in 12 of 75 papaya plants 12–15 days after the bombardment (Table 2). The immunodiffusion analysis revealed that leaf extracts from symptomatic papaya plants, after inoculation with p35S-HA mechanically or by particle bombardment, contained an antigen specifically reacting with PRSV antiserum (data not shown). Filamentous virus particles decorated with PRSV antiserum were also observed and were similar to those seen from plants inoculated with in vitro transcript of pT3-HAG (Figure 4).

Discussion

In this investigation, full-length cDNA clones of PRSV were constructed downstream from a bacteriophage T3 RNA polymerase promoter or a CaMV 35S promoter. The transcripts produced either by in vitro transcription driven by the T3 promoter or by in vivo transcription derived from the 35S promoter were proven to be infectious to the systemic host *C. papaya* and the local lesion host *C. quinoa*.

The in vitro transcript from pT3-Xb with 64 extra nonviral nucleotides at the 3' end of PRSV sequence was not infectious. When this 3'-nonviral sequence was trimmed down to 12 nucleotides, the transcript from pT3-HAG became infectious. In potyviruses, the infectious in vitro transcript of TVMV contained one additional nonviral cytidine residue at the 3' end (Domier et al., 1989). PPV had 12 residues (Riechmann et al., 1990). ZYMV had one guanosine residue (Gal-On et al., 1991), and TEV had 4 residues (Dolja et al., 1992). The presence of 12 additional residues after the poly(A) track of the PRSV transcript from pT3-HAG did not interfere with its infectivity. In this respect, it resembles PPV. However, the longer, 64 residue nonviral sequence of the PRSV transcript from pT3-Xb did interfere with its infectivity. It is not clear why the prolonged 3' nonviral sequence has this effect. One possible explanation is that the secondary structure of the extra nucleotides hampers the proper initiation of (-) RNA synthesized from the 3' end of the transcript (Boyer and Haenni, 1994).

As a general rule, nonviral nucleotides at the 5' ends substantially decrease or even abolish infectivity of an in vitro transcript (Boyer and Haenni, 1994). We constructed an infectious transcript of PRSV with the T3 promoter by removing 49 nonviral nucleotides and introducing one extra guanosine residue at its 5' end by site-directed mutagenesis. This is similar to most of the infectious constructs of potyviruses in which the nonviral nucleotide introduced at the 5' end is a guanosine residue (Domier et al., 1989; Riechmann et al., 1990; Gal-On et al., 1991; Dolja et al., 1992). Both T7 and T3 RNA polymerases initiate transcription primarily at guanosine residues (Bailey et al., 1983; Domier et al., 1989), and the benefit of the added guanosine may be higher efficiency for capping.

Transcripts derived from an initial set of cDNA clones with very short (12-nt) poly(A) tails were not infectious for TVMV, while substitution with 3'- terminal fragments containing longer (37- or 96-nt) poly(A) tails resulted in the production of infectious transcripts (Domier et al., 1989). The presence of a long adenosine sequence at the 3' end will help stabilize the RNA transcript and enhance translational efficiency (Jackson and Standart, 1990). Therefore, it seems that there is a threshold length of A residues below which the transcript is not infectious. The poly(A) tail of our PRSV full-length construct comprised only 36 A residues and is shorter than other infectious transcripts of potyviruses (TVMV with 37 or 96, PPV with 100, ZYMV with 66, and TEV with 70–75).

Table 2. Infectivity assay of p35S-Xb and p35S-HA containing the full length cDNA to PRSV HA with a 35S promoter and a NOS terminator.

Inoculation method	No. of papaya plants showing symptoms			Lesions per leaf of *C. quinoa*		
	p35S-Xb	p35S-HA	PRSV	p35S-Xb	p35S-HA	PRSV
Mechanical inoculation	0/250	24/57	30/30	0 [a]	6 [b]	153 [c]
Particle bombardment	0/105	12/75	–[d]	–	–	–

[a]A total of 40 leaves were inoculated.
[b]Average from a total of 24 inoculated leaves.
[c]Average from a total of 12 inoculated leaves.
[d]Not tested.

Although uncapped in vitro transcripts of ZYMV have low infectivity when plants are inoculated via particle bombardment (Gal-On et al., 1995), the presence of a cap (m^7GpppG) is required at the 5' end for optimal infectivity of potyviral transcripts by mechanical inoculation (Domier et al., 1989; Riechmann et al., 1990; Gal-On et al., 1991; Dolja et al., 1992; Gal-On et al., 1995). In this investigation, only the capped transcript of PRSV was infectious, possibly because it increased translation initiation of viral proteins (Contreras et al., 1982) and protected the transcript from exonucleolytic degradation in host cells (Green et al., 1983). Therefore, a 5' m^7GpppG can replace the role of the VPg. This is in agreement with the observations made for a number of other virus possessing a 5' VPg (Riechmann et al., 1990).

For construction of the full-length cDNA clone, the choice of the RNA polymerase promoter is important. The majority of workers use the T7 promoter, sometimes the SP6 promoter, but very few have used the T3 promoter (Boyer and Haenni, 1994). Our construct of the full-length cDNA clone of PRSV was downstream from the T3 promoter, and its in vitro transcript was highly infectious. Grapevine fanleaf nepovirus (Viry et al., 1993) and TVMV (Domier et al., 1989) have also produced infectious transcripts via the T3 promoter.

For many viruses, the constructs of infectious cDNA did not care much about how long the 3' end nonviral nucleotides is because they usually had a NOS terminator downstream from the viral cDNA (Boyer and Haenni, 1994). The addition of the NOS terminator efficiently prevented the synthesis of transcripts longer than the genomic size, which are usually not infectious (Dessens and Lomonossoff, 1993). But the cases of Yamaya et al. (1988) and Gal-On et al. (1995) showed that the construct of the full-length cDNA clones without the NOS terminator still remained infectious when they were introduced into hosts via transgenic approach or by particle bombardment. The reason for p35S-Xb not being infectious might be due to the extra 33 nonviral nucleotides in front of the 5' end of PRSV sequence. However, the full-length cDNAs of BNYVV, BMV, AlMV, and ZYMV containing <40, 12, 8, and 127 nonviral nucleotides at the 5' end, respectively, remained infectious (Commandeur et al., 1991; Mori et al., 1991; Neeleman et al., 1993; Gal-On et al., 1995). In order to exclude the interference of nonviral residues at the 5' end, Maiss et al. (1992), Gal-On et al. (1995), and Fakhfakh et al. (1996) successfully constructed infectious cDNAs of PPV, ZYMV, and PVY without any additional residues between the 35S promoter and the viral 5' end. Our results coupled with these indicate that the construction of a precise 5' end is important for potyvirus for higher infectivity. Another possibility might be that the 64 nonviral nucleotides at the 3' end of the PRSV sequence in p35S-Xb hindered the initiation of (-) RNA synthesis, and the trimming of these residues down to 10 nucleotides in p35S-HA removed such an interference. Finally, the failure of p35S-Xb to be infectious may be due to error(s) in the PRSV nucleotide sequence in this cDNA clone. However, this seems unlikely because PRSV sequences used for the construction of p35S-Xb and p35S-HA had essentially the same origin.

Symptoms of papaya plants and *C. quinoa* inoculated with infectious in vitro and in vivo transcripts of PRSV were not significantly delayed when compared to those induced by the control viral RNA or PRSV. This is different from the other four infectious potyviral transcripts which usually showed a slight lag of 1 to 13 days for symptom development following mechanical inoculation. The infectious in vitro or in vivo transcript derived from the constructed full-length PRSV cDNA in this investigation is the longest infectious transcript in monopartite plant viruses so far recorded. Our in vitro infectious transcript of PRSV also had a higher infection rate of 56% when compared with the infectious transcripts of other potyviruses [TVMV with 5.5% (Domier et al., 1989); PPV with 49% (Riechmann et al., 1990); ZYMV with 11% (Gal-On et al., 1991) and TEV with 27% (Dolja et al., 1992)]. This high infection rate is beneficial in studying the genetic expression and gene functions of PRSV. The higher infection rate and lack of delay in symptom expression may be due to the following: (1) the cDNA clones were derived from a highly infectious viral RNA population used in the preparation, (2) the two large clones of λgt11-AI and λgt11-CI were selected from a cDNA library by reaction with antisera to PRSV viral products, ensuring a correct reading frame for the polyprotein, (3) high efficiency of capping resulted in highly infectious full-length transcripts, and (4) the 12 nonviral residues at the 3' end might have formed a stable secondary structure to avoid digestion by exonuclease and make it easier for polymerase recognition.

Plants mechanically inoculated either with in vitro transcript generated from pT3-HAG or with p35S-HA showed typical PRSV symptoms. However, symptoms on p35S-HA inoculated papaya plants seemed to appear earlier than those on the in vitro transcript inoculated plants. This phenomenon does not agree with the general belief that RNA would translate directly and initiate infection once it entered the cytoplasm. Possibly, this may indicate that the in vitro transcript was more susceptible to degradation after inoculation. Moreover, the concentration of infectious DNA (15–20 μg) was much higher than in the in vitro transcript (2–3 μg) when it was introduced into the papaya plant.

Plants inoculated either mechanically or by biolistic particle delivery system with p35S-HA showed typical PRSV symptoms, but plants inoculated by the later method developed symptoms 3–7 days slower. This is different from the situation reported with ZYMV (Gal-On et al., 1995). The reason for the delay in symptom development was probably due to the low DNA amount transferred into plant cells, because only less than 1 μg DNA per shoot was delivered into a cluster of 15 plants, quite a small quantity when compared with the 15–20 μg per plant used in the mechanical inoculation. Also, because the molecular size of the full-length cDNA is large (about 14 kb), it might easily be agglomerated or fragmented under the bombardment conditions.

Our ability to synthesize biologically active PRSV transcript from the full-length cDNA template is a significant step forward in molecular studies of the functions of PRSV genes and the roles of specific sequences of PRSV RNA in encapsidation, pathogenicity, transmission, replication, and translation. Moreover, the construction of this full-length clone provides an excellent vehicle for long-term preservation of an unchanged virus culture, either stored as plasmid or as transformed bacteria.

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