Detection of Prunus Necrotic Ringspot Virus Serotypes in Herbaceous and *Prunus* Hosts with a Complementary RNA Probe

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ABSTRACT

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Complementary DNAs (cDNAs) prepared from genomic RNA of a peach isolate of Prunus necrotic ringspot virus (PNRSV) were used to produce a ^{32}P-labeled complementary RNA (cRNA) probe that was capable of detecting PNRSV in tissue extracts. RNA transcripts of an 800 bp cDNA fragment inserted into plasmid pGEM-7Zf(+) were obtained using SP6 and T7 RNA polymerases. Dot-blot hybridizations using radiolabeled SP6 transcripts were compared to enzyme-linked immunosorbent assay (ELISA) for the detection of PNRSV serotypes in peach, cherry, and herbaceous hosts. In most tissues the limits of detection of PNRSV were similar with ELISA and cRNA hybridization. However, PNRSV serotype CH30 reacted poorly in ELISA but was readily detected by the cRNA probe. The probe did not detect prune dwarf, apple mosaic, or tobacco streak ilarviruses or a virus isolated from hops previously considered to be PNRSV.

Additional keyword: riboprobe

Prunus necrotic ringspot ilarvirus (PNRSV) (5,7,13) is a widespread and serious pathogen of many Prunus species including peach (*P. persica* L. Batsch.) and sweet cherry (*P. avium* L.) (17,18, 21,26). The virus occurs as numerous strains, isolates, or biotypes that vary widely in their pathogenic, biophysical, and serological properties (3,4,11,17).

Serological methods such as the enzyme-linked immunosorbent assay (ELISA) have been widely used for detection of PNRSV in *Prunus* species (15,16,21,26). However, the discovery of three distinct serotypes of PNRSV in sweet cherry, designated CH3, CH9, and CH30 (17), may complicate the identification of PNRSV infections solely on the basis of serology. The CH30 serotype reacts weakly in ELISA tests performed using antibodies to CH9 serotypes (17).

Recently, radiolabeled RNA transcripts complementary to specific nucleic acid sequences (cRNA or riboprobes) have been utilized as probes for detection of plant (1,9,20,29) and animal viruses (25) and viroids (8,23,27). The cRNA transcripts can be labeled to high specific activity, and the hybridization reaction can be carried out under stringent conditions that improve sensitivity and specificity of the hybridization (14,23, 24,29).

This research evaluates the usefulness of a radioactively labeled cRNA probe for the detection of diverse serotypes of PNRSV in tissues of peach, sweet cherry, *Chenopodium quinoa* Willd., and cucumber (*Cucumis sativus* L.), and compares this technique to the more commonly used ELISA.

MATERIALS AND METHODS

Virus isolates. Virus isolates and host plants used in these investigations are listed in Table 1. Most isolates of PNRSV were originally transmitted from sweet cherry. Other isolates of PNRSV originated in peach (PE5, NRS-S, PDV-S), rose (RO2), almond (AL14), sour cherry (G), and hop (NRSV-Hop). Isolates of prune dwarf virus (PDV) (6) were from sweet cherry and peach, apple mosaic virus (ApMV) from apple, and tobacco streak virus (TSV) from white sweetclover (*Melilotus alba* Medikus). Tissues from peach trees graft-inoculated with sweet cherry or peach isolates or naturally infected field-grown trees were used in some experiments. The PDV isolates were included in these tests because PDV and PNRSV are often found as mixed infections in *Prunus*. Peach cultivars included Suncrest, Sunhigh, Red Haven, Halbrite, and J.H. Hale. Sweet cherry tissues (cv. Bing) were

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obtained from G.I. Mink, Prosser, Washington.

ELISA. Triple-antibody sandwich ELISA was performed on cherry, peach, cucumber, and *C. quinoa* tissues infected with various isolates of PNRSV. Polystyrene microtiter plates were coated 2 hr at 37 C with 1 μg/ml of immunoglobulin (Ig) G purified from rabbit polyclonal antiserum, obtained from G. I. Mink, which had been prepared using isolate CH61 as the immunogen. Tissues were triturated in 10 volumes of phosphate buffered saline, pH 7.3, containing 0.05% (v/v) Tween 20, 2% (w/v) polyvinyl pyrrolidone, 0.2% (w/v) ovalbumin, 0.45% (w/v) sodium diethyldithiocarbamate (PEP-Dieca buffer) (15), and incubated overnight at 4 C. Tenfold dilutions of triturates were prepared in PEP-Dieca buffer. After washing, plates were incubated 1 hr at 22–24 C with monoclonal antibody NA70C9 (American Type Culture Collection [ATCC] PVAS 604)(10) diluted 1:3,000 in PEP buffer. Finally, goat anti-mouse IgG-alkaline phosphatase conjugate was diluted 1:2,500 in PEP buffer and incubated 3 hr at 37 C. The enzyme substrate was *p*-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine, pH 9.8.

Indirect ELISA of cherry and peach tissues for PDV was conducted by first incubating triturates, prepared as described above, in polystyrene microtiter plates for 1 hr at 37 C. Plates were then washed, incubated 1 hr at 37 C with a 1:2,000 dilution of antiserum to a Washington isolate of PDV provided by G. I. Mink, washed again, incubated 3 hr at 37 C with a 1:2,500 dilution of goat anti-rabbit IgG-alkaline phosphatase conjugate, and developed with *p*-nitrophenyl phosphate substrate.

In both the PNRSV and PDV tests, all reagent volumes were 100 μl per well. Plant extracts were loaded into duplicate wells. Absorbance values at 405 nm were measured with a spectrophotometer 2 hr after addition of substrate for *Prunus* bud tissues, cucumber, and *C. quinoa*, and after 18 hr for *Prunus* leaf tissues. The experiments were repeated at least once.

RNA isolation and molecular cloning. Viral nucleoproteins of isolate PE5 were purified from systemically infected *C. quinoa* as described (4,19) and dissociated for 1 hr at 55 C in a solution containing 1% sodium dodecyl sulfate (SDS) and 250 μg/ml proteinase K. After extraction in phenol-chloroform, viral RNAs were precipitated in sodium acetate and ethanol (24) and resuspended in water.

Complementary DNA (cDNA) synthesis was primed with random hexamers and performed using a commercially available kit (Riboclone, Promega Corp., Madison, Wisconsin). The cDNAs were blunt-end ligated into the *Sma*I restriction site of plasmid pUC19 and used to transform competent *Escherichia coli* DH5α cells (24). Recombinant colonies were selected on the basis of hybridization to a ^{32}P-labeled first-strand cDNA probe prepared from purified RNA 3 of PNRSV isolate CH57 isolated by gel electrophoresis. The cDNA inserts in recombinant plasmids were excised by *Eco*RI and *Bam*HI digestion and evaluated for size by agarose gel electrophoresis. The largest inserts were electroeluted from gel slices and subcloned into the *Eco*RI-*Bam*HI sites of plasmid pGEM-7Zf(+) (Promega), which contains opposing SP6 and T7 RNA polymerase promoters. Plasmids containing an 800-bp cDNA fragment, designated pJC5-20, were linearized with *Eco*RI or *Bam*HI for transcription by SP6 or T7 RNA polymerases, respectively. Radioactively labeled cRNA transcripts of pJC5-20 were obtained using ^{32}P-uridine 5′ triphosphate as the labeled nucleotide (14,24).

Preparation of tissue extracts for dot blots. Dormant buds or young leaves were obtained in March or May, respectively, from sweet cherry and peach trees. Samples consisted of tissues taken from various parts of the trees to minimize effects of uneven virus distribution. Leaf tissues from systemically infected *C. quinoa* or cucumber were obtained 7–10 days after inoculation (4) with the various ilarviruses (Table 1). Tissues were triturated in 10 volumes of 200 mM dibasic potassium phosphate containing 0.1% Triton X-100, 5 mM dithiothreitol, and 10 mM 2-mercaptoethanol (27). Triturates were centrifuged briefly, and a 200-μl aliquot of the supernatant was removed, added to 200 μl of denaturation solution (8× SSC + 10% formaldehyde; 1× SSC is 150 mM NaCl, 15 mM Na citrate, pH 7.0), and heated to 60 C for 15 min. These preparations are referred to as crude extracts. Tenfold dilutions of crude extracts were prepared using 4× SSC + 5% formaldehyde as diluent. For preparation of nucleic acid precipitates, crude extracts were emulsified with an equal volume of phenol-chloroform, and the nucleic acids precipitated from the aqueous phase with sodium acetate and ethanol. After incubation at −20 C and centrifugation, the precipitated nucleic acids were resuspended in 50–100 μl 10 mM Tris, 1 mM EDTA buffer (TE), pH 7.5. A 10-fold dilution series of nucleic acid precipitates was prepared using TE as diluent.

Dot-blot hybridization. Aliquots (2 μl) of the above dilution series were spotted onto 20× SSC-equilibrated nitrocellulose membranes and baked at 80 C under vacuum for 1.5–2 hr. Membranes were prehybridized for 1 hr at 55 C in 50% formamide, 25 mM sodium cacodylate buffer, pH 7.0, 22 mM NaCl, 1.2 mM EDTA, 0.1% SDS, and 0.5 mg/ml yeast tRNA. Dextran sulfate was added to a final concentration of 10%, and the ^{32}P-labeled cRNA transcript of linearized pJC5-20 was added to a concentration of about 200,000–500,000 cpm per milliliter of hybridization solution. Membranes were hybridized overnight at 55 C, rinsed twice in 2× SSC, and incubated 15 min at room temperature in 2× SSC containing 1 μg/ml RNase A. The final wash was in 0.1× SSC, 0.1% SDS for 30 min at 65 C. Membranes were exposed to X-ray film with an intensifying screen at −70 C for 24–72 hr.

Table 1. Designation, serotype, and source of ilarvirus isolates used in ELISA and cRNA detection assays

Isolate	Synonym	Serotype[a]	Host[b]	Source[c]	Reference
PNRSV					
PE5	SC1	CH9	Cq	S. W. Scott	3, 4
PV22	G	CH9	Cs	ATCC	3, 4, ATCC
CH25	G	CH9	Sc	G. I. Mink	3, 4, 17
CH30	...	CH30	Cq	G. I. Mink	3, 4, 17
CH57	...	CH9	Cq, Sc, P	G. I. Mink	3, 4
CH59	...	CH9	Sc	G. I. Mink	...
CH61	...	CH9	Sc, P	G. I. Mink	3, 4, 17
CH71	...	CH30	Sc	G. I. Mink	3, 4
CH133	...	CH9	Cq	J. K. Uyemoto	3, 4
RO2	...	CH9	Cq	J. M. Crosslin	3, 4
AL14	...	CH9	Cq	J. K. Uyemoto	3, 4
NRS-S	...	ND	P	S. W. Scott	...
NRSV-Hop	HP-2	Hop	Cs	C. B. Skotland	4, 28
PDV[c]					
CH60	...	NA	Sc, P	G. I. Mink	...
PDV-S	...	NA	P	S. W. Scott	...
Other[e]					
ApMV	PV32	NA	Cs	G. I. Mink	ATCC
TSV	Mel 40	NA	Cq	W. J. Kaiser	12

[a]Serotype of Prunus necrotic ringspot virus (PNRSV) as determined previously (3,4,17). NA = not applicable, ND = not determined.
[b]Cq = *Chenopodium quinoa*, Cs = *Cucumis sativus*, Sc = sweet cherry, P = peach.
[c]ATCC = American Type Culture Collection.
[d]Prune dwarf virus.
[e]ApMV = Apple mosaic virus, TSV = tobacco streak virus.

RESULTS

ELISA. Most isolates of PNRSV were readily detected in tissues of *C. quinoa* and in cherry and peach tissues collected in March (buds) or in May (leaves) (Fig. 1). Dilution end points were typically 10^{-2} to 10^{-3}. Isolates CH30 and CH71, which are CH30 serotype (4,17) produced very low absorbance values in these ELISA tests and have consistently produced low absorbance values in numerous similarly conducted ELISA tests performed over the past several years (Crosslin, *unpublished*).

Specificity of the ELISA procedures was confirmed when extracts of cucumber or *C. quinoa* infected with ApMV, TSV, or NRSV-Hop failed to produce A_{405} values greater than healthy controls. Cucumber infected with the G isolate of PNRSV (ATCC PV22) produced absorbance values similar to those presented for the CH57 isolate in *C. quinoa* tissues (*data not shown*).

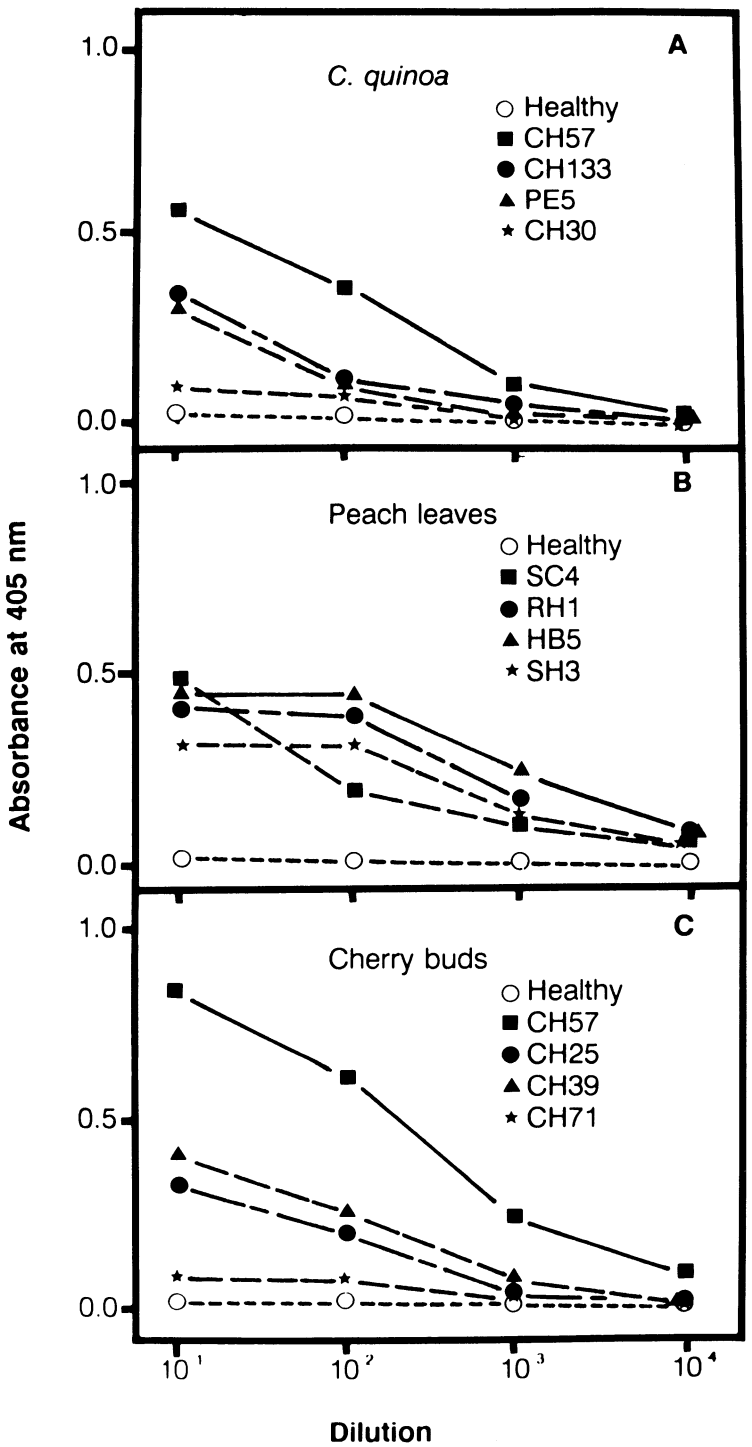

Fig. 1. Detection of Prunus necrotic ringspot virus in peach, sweet cherry, and *Chenopodium quinoa* tissues by triple-antibody sandwich enzyme-linked immunosorbent assay (ELISA). **(A)** Systemically infected *C. quinoa*; **(B)** peach leaves collected from field trees in May; and **(C)** sweet cherry buds collected in March. Absorbance values were recorded after 2 hr **(A and C)** or 18 hr **(B)**.

Hybridization of SP6 and T7 cRNA transcripts. Preliminary experiments showed that cRNA transcripts of linearized pJC5-20 transcribed using T7 RNA polymerase failed to hybridize with extracts from PNRSV-infected tissues, purified virus, or viral RNA (*data not shown*), suggesting that the T7 transcript is viral sense in polarity. Transcripts of pJC5-20 obtained with SP6 RNA polymerase, however, hybridized with similar samples (Figs. 2–6) indicating that the SP6 transcript is viral antisense. SP6 transcripts were used as the probe in all subsequent experiments.

Purified RNA and virus. In tests with purified RNA 3 of isolate PE5, the limit of detection by the probe was about 5 pg of RNA (Fig. 2). Fifty to 500 pg of purified nucleoproteins of PNRSV isolate CH133 could be detected with the probe (*data not shown*).

Herbaceous tissues. The probe hybridized with crude extracts prepared from *C. quinoa* infected with each of the six PNRSV isolates, including isolate CH30 (Fig. 3). The virus was detected in extracts from infected plants diluted 10^{-3} to 10^{-4} but not in extracts from healthy *C. quinoa*. Cucumber plants infected with PNRSV isolate PV22 reacted similarly (*data not shown*). The probe failed to hybridize with crude extracts prepared from cucumber or *C. quinoa* tissues infected with ApMV, TSV, or NRSV-Hop (*data not shown*).

Peach tissues. Dilutions (10^{-2} to 10^{-3}) of crude extracts and nucleic acid precipitates from infected peach leaves hybridized with the PNRSV-specific probe (Figs. 4 and 5). Extracts from trees free of PNRSV (trees SC2, CH60, and PDV-S), as determined by ELISA, showed no hybridization. Extracts from trees infected with PDV isolates CH60 or PDV-S did not hybridize with the probe (Fig. 5).

Cherry tissues. Nucleic acid precipitates from dormant sweet cherry buds infected with PNRSV also showed hybridization with the probe (Fig. 6). Isolate CH71, which is a CH30 serotype, reacted as strongly as the other isolates, which are of the CH9 serotype (4). Dilution end points were about 10^{-2}.

DISCUSSION

Although ELISA has been used by a number of researchers for detection of PNRSV in *Prunus* species (11,17,21,26), it seems possible that some infections could be missed because of poor reactivity of a given isolate with the antibodies employed in the test. The results described here and those of other researchers (17) have demonstrated that plant extracts prepared from tissues infected with PNRSV isolates of the CH30 serotype react poorly in tests conducted with antisera to Fulton's G

isolate or serologically similar isolates. Also, purified nucleoproteins of isolate CH30 produced low absorbance values in ELISA tests utilizing antisera to Fulton's G isolate when compared with equivalent concentrations of nucleoproteins of CH9 serotype isolates (17). These results indicate that the low ELISA values produced by CH30 serotype isolates did not result from lower virus titers in plants infected with these isolates in comparison with CH9 serotype isolates.

The CH3 serotype of PNRSV, which is more closely related to the CH9 serotype than is the CH30 serotype, is readily detected by ELISA (17) and was detected in cherry extracts by the probe used in this study (*data not shown*).

Halk et al (10) reported detection of several PNRSV isolates, including CH30 and NRSV-Hop, in ELISA using monoclonal antibody NA70C9. However, they employed indirect ELISA procedures, and purified nucleoproteins were used as test antigen in their experiments (10). These differences in procedures may account for the low absorbance values observed with CH30 serotypes and the failure to detect NRSV-Hop in our experiments.

In contrast to the results obtained by ELISA, the cRNA probe readily detected two CH30 serotype isolates: CH30 in *C. quinoa* and CH71 in sweet cherry. Detection of these two isolates by the cRNA probe was similar to that of the other PNRSV isolates transmitted from rosaceous hosts, which are classified as CH9 serotype (4). Low absorbance values were obtained in ELISA on tissues infected with CH30 serotypes of PNRSV. Based solely on these results from ELISA, these plants might be considered free of PNRSV. However, there were no such ambiguous results with *Prunus* bud or leaf samples in dot blots.

The cRNA probe detected PNRSV isolates that had been originally transmitted from peach, cherry, rose, and almond, but failed to detect the isolate from hops. Although NRSV-Hop was considered to be an isolate of PNRSV by Smith and Skotland (28), this isolate shows serological relatedness to ApMV, which is an attribute not shared by any of the PNRSV isolates obtained from rosaceous hosts (3,4). Also, Halk et al (10) found that NRSV-Hop reacted similarly to ApMV when tested against ApMV-specific monoclonal antibodies in indirect ELISA. Certain isolates with serological properties similar to NRSV-Hop have been termed intermediate in their relationship to PNRSV and ApMV by some researchers (2). Additionally, isolate NRSV-Hop also possesses biophysical properties that indicate that it is indeed quite different from the other PNRSV isolates tested (3,4) and might be more correctly called a strain of ApMV.

Varveri et al (29) reported that plum pox virus (PPV) could be detected in apricot tissues using a cRNA probe. These researchers found that, compared with ELISA, the probe was 10–250 times more sensitive in detecting PPV, depending upon the test conditions. Similarly, Powell et al (20) noted improved detection of tomato ringspot virus using a cRNA probe. Our results, however, indicate that ELISA and dot-blot hybridization tests with CH9 serotypes show similar levels of sensitivity. It may be possible to improve sensitivity by concentrating nucleic acids from a larger volume of tissue extract and/or spotting a larger volume of each sample onto the membranes. Additionally, the proce-

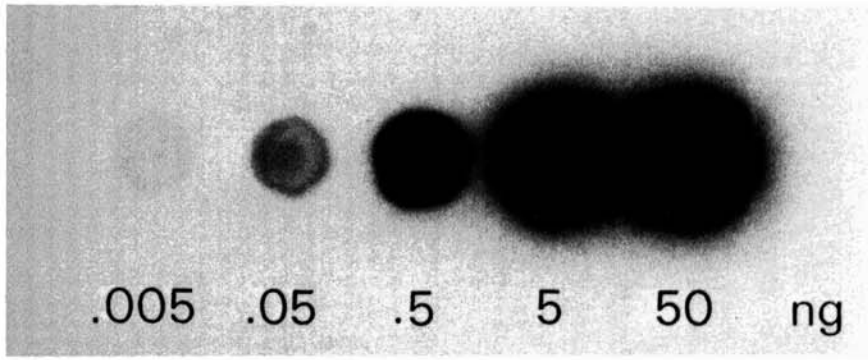

Fig. 2. Dot-blot hybridization of agarose gel-purified RNA 3 of PNRSV isolate PE5 with a ^{32}P-labeled cRNA probe transcribed from pJC5-20. Dilutions were prepared in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

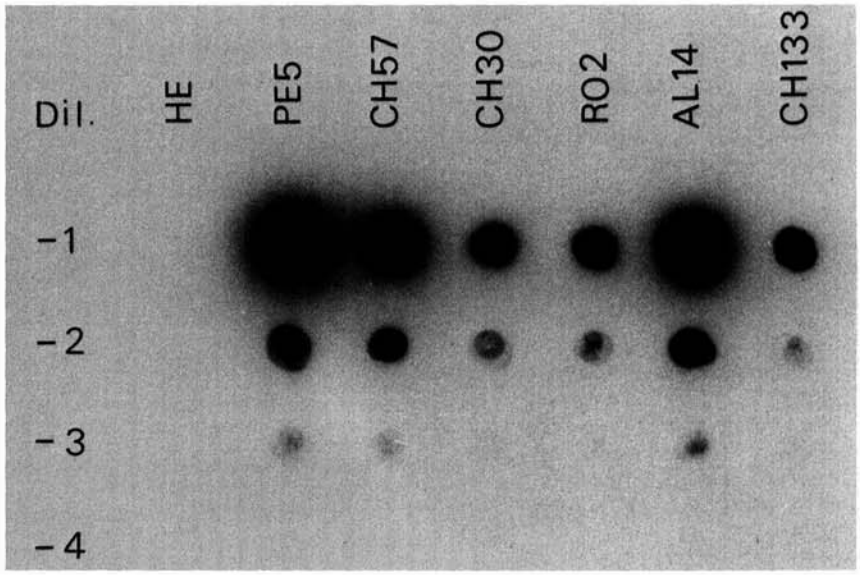

Fig. 3. Dot-blot hybridization of *Chenopodium quinoa* crude extracts with ^{32}P-labeled cRNA probe from plasmid pJC5-20. Isolate designations as in Table 1. He = healthy *C. quinoa*. Tenfold dilutions prepared in 4× SSC, 5% formaldehyde.

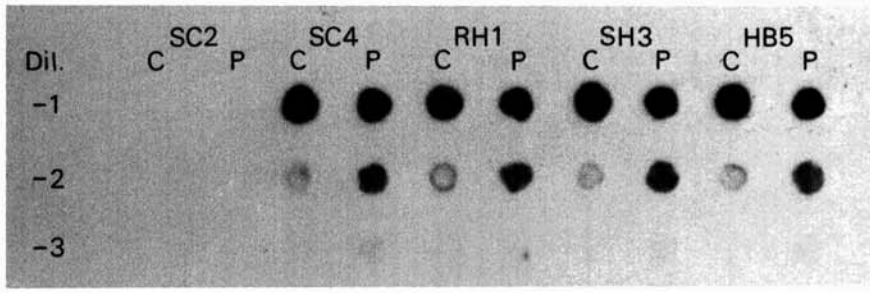

Fig. 4. Dot-blot hybridization of the cRNA probe with crude leaf extracts (C) and nucleic acid precipitates (P) from a healthy peach tree (SC2) or trees naturally infected with PNRSV (SC4, RH1, SH3, HB5). Tenfold dilutions of crude extracts were prepared in 4× SSC, 5% formaldehyde. Nucleic acid precipitates were diluted in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

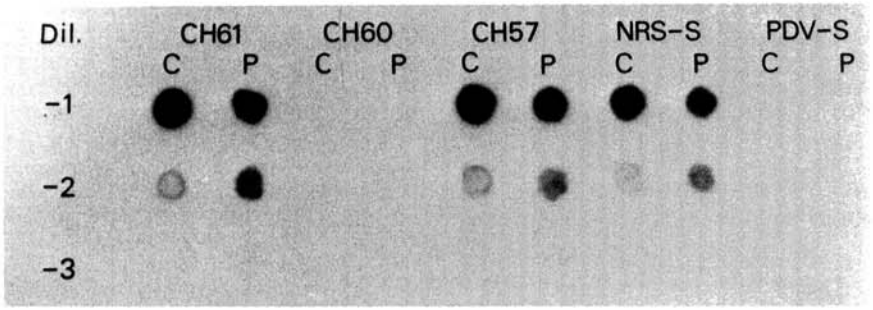

Fig. 5. Dot-blot hybridization of the cRNA probe with crude leaf extracts (C) and nucleic acid precipitates (P) from peach trees graft-inoculated with sweet cherry (CH61 and CH57) or peach (NRS-S) isolates of PNRSV or PDV isolates CH60 and PDV-S from sweet cherry and peach, respectively. Tenfold dilutions of crude extracts were prepared in 4× SSC, 5% formaldehyde.

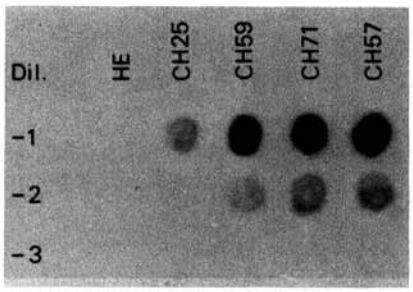

Fig. 6. Dot-blot hybridization of the cRNA probe with nucleic acid precipitates from dormant sweet cherry buds. Isolate designations as in Table 1. He = healthy. Tenfold dilutions of crude extracts were prepared in 4× SSC, 5% formaldehyde.

dures utilized herein to prepare tissue extracts, although effective, may not be optimal for recovery of viral RNA from all types of tissues. Indeed, early experiments performed using several extraction buffers (*data not shown*) indicated that the buffer has a marked effect on the hybridization results. It proved particularly difficult to obtain good hybridization results with cherry tissues. Other workers (22) have reported difficulty in extracting nucleic acids from woody hosts. Direct tissue blotting may circumvent this problem and has been somewhat successful with *C. quinoa* tissues (*data not shown*) but has not yet been used with *Prunus* samples.

The ability to detect diverse PNRSV serotypes in herbaceous and woody hosts with a single cRNA probe should be useful in nursery certification programs, in plant quarantine, and in studies of relationships among PNRSV isolates.

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