Detection of Andean Potato Virus X Isolates by Radioactive and Nonradioactive Nucleic Acid Spot Hybridization Tests

M. Querci, L. F. Salazar, and E. N. Fernandez-Northcote

International Potato Center, Apartado 5969, Lima, Peru.

We thank R. Hammond (USDA-ARS, Beltsville, MD) for advice in the cloning and sequencing procedures, and J. Hammond and R. A. Owens (USDA-ARS, Beltsville, MD) for the critical review of the manuscript.
Accepted for publication 17 September 1992.

ABSTRACT

Querci, M., Salazar, L. F., and Fernandez-Northcote, E. N. 1993. Detection of Andean potato virus X isolates by radioactive and nonradioactive nucleic acid spot hybridization tests. Phytopathology 83:171-176.

A cDNA probe, pX61, prepared from the Andean potato virus X (PVX) cp strain was tested against a broad spectrum of Andean PVX isolates grouped in two serotypes: 1) the PVX^A Andean serotype detected only in Peru and Bolivia, which includes the cp and HB strains; and 2) the PVX^O common serotype, which contains isolates serologically similar to those occurring elsewhere in the world. In radioactive nucleic acid spot hybridization tests (R-NASH) of virus in crude sap using the ^{32}P-labeled RNA probe pX61, the PVX^A isolates showed stronger hybridization signals and a detectability usually from two to six threefold dilution steps higher than those shown for PVX^O isolates. The difference in the detectability of isolates from PVX^A and PVX^O serotypes was similar to that in nonradioactive nucleic acid spot hybridization tests (NR-NASH) with biotinylated DNA probe pX61. However, detectability in NR-NASH was lower than in R-NASH. When biotinylated DNA probes of pX61 (PVX^A-specific) and pPVX19 (PVX^O-specific), prepared from a British PVX isolate of the common European strain-group 3, were compared in NR-NASH, pPVX19 hybridized much more strongly to most isolates from the PVX^O serotype than to those from the PVX^A serotype. Several PVX^O isolates, mostly from Bolivia, reacted as weakly with pPVX19 as the PVX^A isolates. For R-NASH and NR-NASH tests, virus concentration in crude sap was checked by the double-antibody sandwich form of enzyme-linked immunosorbent assay, confirming that the differences obtained were not because of differences in virus concentration. These results were reconfirmed in R-NASH tests with DNA probes pX61 and pPVX19 using known concentrations of purified RNA from selected isolates. The differences shown between PVX^A and PVX^O isolates stress the importance of using the appropriate probes to detect PVX in breeding programs for resistance and quarantine purposes.

RESUMEN

El probador de ADNc pX61 preparado usando el strain PVXcp, se utilizó contra un espectro amplio de aislamientos andinos de PVX agrupados en dos serotipos, el andino PVX^A detectado únicamente en el Perú y Bolivia y que incluye a los strains cp y HB y el común PVX^O que incluye aislamientos serológicamente similares a los que ocurren en otras partes del mundo. En pruebas de hibridación local de ácidos nucleicos modalidad radioactiva (R-NASH) con virus en savia de hojas y con el probador de ARN pX61 marcado con ^{32}P, se obtuvo una señal de hibridación más fuerte y una detectabilidad generalmente de dos a seis pasos más altos en diluciones de la savia 1:3, en el caso de los aislamientos PVX^A que en el de los aislamientos PVX^O. La diferencia en la detectabilidad entre los serotipos PVX^A y PVX^O, fue similar en pruebas de hibridación local de ácidos nucleicos modalidad no-radioactiva (NR-NASH) usando el probador de ADN pX61 biotinilado, sin embargo la sensibilidad fue menor en NR-NASH que en R-NASH. Cuando se comparó en NR-NASH probadores de ADN biotinilados de pX61, y de pPVX19 preparado usando un aislamiento británico de PVX del grupo de strains-3 común en Europa, la mayoría de los aislamientos del serotipo PVX^O reaccionaron mas fuertemente que los del serotipo PVX^A con el probador pPVX 19. Varios aislamientos PVX^O generalmente provenientes de Bolivia también reaccionaron debilmente con el probador pPVX19. La concentración apropiada de virus para las pruebas de R-NASH y NR-NASH con savia de hojas se chequeó por DAS-ELISA confirmando de que las diferencias obtenidas no dependian de diferencias en la concentración de virus. Estos resultados se confirmaron en R-NASH con los probadores de ADN pX61 y pPVX19 utilizando concentraciones definidas de ARN purificado de aislamientos selectos. Las diferencias encontradas entre los aislamientos PVX^A y PVX^O enfatizan la importancia del uso de probadores apropiados para la detección de PVX en programas de mejoramiento para resistencia y propósitos cuarentenarios.

Additional keywords: dot blot hybridization, potato, potexvirus variability.

The nucleic acid spot hybridization (NASH) technique (also referred to as dot blot hybridization) has been reported for the detection of potato X (PVX), potato Y (PVY), and potato leafroll (PLRV) viruses using radioactively labeled complementary DNA (cDNA) probes (2,4,19,21), as well as for potato spindle tuber viroid (PSTVd) in potato, using radioactively labeled RNA probes (20). Nonradioactive, biotinylated RNA and DNA probes for the detection of PVX and potato S (PVS) viruses in crude potato extracts have also been reported (6,11). Other nonradioactive systems of nucleic acid labeling and detection, including luminescent DNA probes and luminography, were compared in the detection of purified PVX, PVY, and PVS (1). They were also compared in the detection of purified PSTVd and PSTVd in tomato leaf extracts (13). Baulcombe and Fernandez-Northcote (3) evaluated the ability of cDNA probes, prepared from a British PVX isolate of the common European strain (strain-group 3) and from a British common strain of PVY^O, to detect distinct PVX strains and a broad spectrum of PVY isolates, respectively. The two PVY probes used in that work did not detect differences among the PVY isolates from the three groups of strains (PVY^O, PVY^N, and PVY^C). However, PVX probes showed a stronger reaction with the non-Andean strains than with the Andean strains cp and HB. In the present work, a cDNA probe, (pX61), synthesized from the cp strain, was tested as a probe for detection of a broad spectrum of Andean PVX isolates selected from two serotypes (8): 1) the PVX^A Andean serotype, detected only in Peru and Bolivia, which includes the cp and HB strains; and 2) the PVX^O serotype, which groups isolates that are serologically similar with those occurring elsewhere in the world. The pX61 was evaluated and compared with Baulcombe's pPVX19 in both radioactive (R) and nonradioactive (NR) NASH tests. The cDNA inserts of both probes were sequenced.

MATERIALS AND METHODS

Isolates and spotting. Isolates and strains of PVX used in the experiments are listed in Tables 1 and 2. Strains cp and HB (17) were kindly donated by C. Fribourg (Universidad Nacional Agraria La Molina, Lima, Peru) and maintained at the International Potato Center (CIP). The cp and HB strains were originally isolated from the high Andes of central-southern Peru and southern Bolivia, respectively. All isolates and strains were maintained in *Nicotiana glutinosa* L. grown in a greenhouse at 18–24 C. Crude leaf sap from healthy and infected plants was extracted using a roller press and microfuged at 9,880 *g* for 5 min. Threefold dilutions of the supernatant were prepared in 1.5 M NaCl plus 0.15 M trisodium citrate, pH 7.0 (10× SSC). Three microliters of each crude sample or known concentrations of purified viral RNA in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE buffer) were applied to 0.45-μm nitrocellulose filters (Schleicher and Schuell) previously wetted for 5 min in distilled water and then equilibrated in 10× SSC for 5 min. The filters were then air-dried and baked under vacuum at 80 C for 2 h.

Estimation of PVX titer by enzyme-linked immunosorbent assay (ELISA). An aliquot from the same crude sap samples used for NASH was taken for use in ELISA. Threefold dilutions were prepared in phosphate-buffered saline containing 0.05% Tween-20, 2% polyvinylpyrrolidone (M_r 44,000), and 1% egg albumin. The direct double-antibody sandwich form of ELISA (DAS-ELISA) was used, as described by Clark and Adams (5), and 200 μl of each diluted sample was added to each well of the ELISA plate. The coating immunoglobulin G (IgG) was prepared from the PVXcp isolate, and the detecting IgG, conjugated to alkaline phosphatase, was prepared from the PVX_{HB} isolate.

Virus purification and viral RNA extraction. The Andean PVXcp isolate, used for the preparation of probe pX61, and the other PVX isolates used in this work were purified from infected *N. glutinosa* leaves. Frozen leaves were homogenized in 0.1 M phosphate buffer, pH 8.0, containing 0.2% 2-mercaptoethanol and 10% ethanol. The homogenate was treated with 1% Triton X-100 at 4 C for 1 h, precipitated with 0.2 M NaCl, 4% polyethylene glycol 6,000–8,000, for 1 h at room temperature, and centrifuged at 7,840 *g*. The pellets were resuspended in 0.05 M phosphate buffer, pH 8.0, and subjected to centrifugation through a 30% sucrose cushion (6 ml per tube) at 69,231 *g* for 150 min, followed by centrifugation through a linear sucrose density gradient (10–40%) in 0.05 M phosphate buffer, pH 7.2. Fractions

TABLE 1. Identity of isolates used in this work other than those cited in Table 2.

Isolate	Geographic Origin	Serotype (Serogroup)
73	Huanuco, Peru	X^O (I/II)
D-CF[a]	Peru	X^O (IV)
Luc 3-1	Huancayo, Peru	X^O (IV)
Luc 5-2	Huancayo, Peru	X^O (IV)
Luc 6-1	Huancayo, Peru	X^O (IV)
Luc 9	Huancayo, Peru	X^O (IV)
Luc 10	Huancayo, Peru	X^O (IV)
65	Huanuco, Peru	X^O (IV)
69	Huanuco, Peru	X^O (IV)
57CH	Chile	X^O (IV)
85CH	Chile	X^O (IV)
119CH	Chile	X^O (IV)
GUA2[b]	Guatemala	X^O (IV)

[a] Provided by C. Fribourg (Universidad Nacional Agraria, La Molina, Lima, Peru).
[b] Provided by J. Abad (International Potato Center, Lima, Peru.)

TABLE 2. Reaction of selected Potato Virus X (PVX) isolates in DAS-ELISA, and in NASH tests using RNA probe pX61 and DNA probes pPVX19 and pX61 prepared from a PVX^O and a PVX^A isolate.

Isolate	Geographic Origin	Serotype (Serogroup)	DAS-ELISA[a]	R-NASH[b]	NR-NASH[c]	
				pX61	pPVX19	pX61
Experiment A						
2	Cuzco, Peru	X^O (I/II)	>10[d]	5	6	4
3	Cuzco, Peru	X^O (IV)	>10	6	6	4
8	Cuzco, Peru	X^O (IV)	9	7	6	4
2Bo	Boqueron, Bol.	X^O (IV)	>10	6	3	4
3Bo	Boqueron, Bol.	X^O (IV)	6	6	5	5
HB	Bolivia	X^A(III)	7	8	2	6
cp	Peru	X^A(III)	>10	>10	2	8
Healthy			0	0	0	0
Experiment B						
20	Cuzco, Peru	X^O (I/II)	8	5	1	3
46Bo	Huatajata, Bol.	X^O (I/II)	10	4	1	4
73Bo	Cochabamba, Bol.	X^O (I/II)	9	5	1	4
14	Cuzco, Peru	X^O (IV)	8	4	4	3
59	Huanuco, Peru	X^O (IV)	6	4	4	2
38Bo	Chirapaca, Bol.	X^O (IV)	5	5	3	3
HB	Bolivia	X^A (III)	9	9	1	7
cp	Peru	X^A (III)	9	>10	1	8
Healthy			0		0	0
Experiment C						
2	Cuzco, Peru	X^O (I/II)	8	9	8	0
27Bo	Chulchulcani, Bol.	X^O (I/II)	>10	5	1	1
20Bo	Montepunto, Bol.	X^O (I/II)	>10	8	1	3
73Bo	Cochabamba, Bol.	X^O (I/II)	9	8	1	2
8	Cuzco, Peru	X^O (IV)	9	4	5	0
38Bo	Chirapaca, Bol.	X^O (IV)	>10	9	4	1
HB	Bolivia	X^A (III)	>10	10	1	3
cp	Peru	X^A (III)	>10	>10	1	9
Healthy			0	0	0	0

[a] Double-antibody sandwich form of ELISA.
[b] Radioactive nucleic acid spot hybridization technique.
[c] Nonradioactive nucleic acid spot hybridization technique.
[d] Numbers assigned to reciprocal (× 1,000) of highest dilutions of sap extracted from *Nicotiana glutinosa* leaves healthy or infected with PVX isolates, at which a reaction was noted after visual observation: 1 = 0.08, 2 = 0.24, 3 = 0.73, 4 = 2.19, 5 = 6.56, 6 = 19.7, 7 = 59.1, 8 = 177, 9 = 531, 10 = 1,594.

containing virions were further concentrated by ultracentrifugation and resuspended in TE buffer. For RNA extraction (9), 0.2–0.5 mg/ml of purified virus in TE buffer was incubated for 30 min at 37 C with 50 μg/ml of proteinase K in the presence of 0.1% sodium dodecyl sulfate (SDS). The mixture was then extracted once with TE buffer-saturated phenol (1:1 w/v), extracted twice with phenol/chloroform (1:1), and extracted once with chloroform. The RNA was concentrated by ethanol precipitation and resuspended in sterile diethyl pyrocarbonate-treated distilled water (15).

cDNA synthesis and cloning. PVXcp RNA was used for cDNA preparation. First-strand synthesis was obtained using M-MLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD [BRL]) by priming the RNA with either oligo dT_{12-18} (10) or a random hexa-nucleotide mixture (pdN_6) (both from Pharmacia). After RNA hydrolysis, second-strand cDNA synthesis was carried out using the Klenow fragment of DNA Polymerase I (BRL) by standard procedures (15).

Double-stranded cDNA was analyzed by gel electrophoresis, and aliquots were digested with three different restriction enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII). The fragments were ligated into pSP64 plasmid that was separately digested with the same restriction enzymes. Recombinant plasmids introduced into *Escherichia coli* JM83 were selected by colony hybridization (15). The presence and size of inserts was determined by plasmid isolation (15) followed by restriction enzyme digestion and agarose gel electrophoresis with DNA size markers. The pX61 clone selected for this work contains a 1,100-bp *Hin*dIII cDNA fragment inserted into the *Hin*dIII site of plasmid pSP64.

Probe pPVX19. Probe pPVX19 (3), prepared from a British PVX isolate of the common European strain-group 3, was donated by D. C. Baulcombe. The probe was a 937-bp cDNA inserted in plasmid pBR322 (4).

DNA sequencing. The nucleotide sequences of cDNA clones pX61 and pPVX19 were obtained by the dideoxy chain termination method (23) using the Taq Track sequencing system from Promega (Madison, WI). The genome coordinates of the two probes were determined by comparison with the sequences reported by Huisman et al (12) for PVX strain X3, and Orman et al (18) for PVX strain cp.

R-NASH. A ^{32}P-labeled RNA probe was prepared essentially according to the procedure described by Melton et al (16) using a commercial kit (Promega). Plasmid pSP64:pX61 was linearized with *Eco*RI, extracted twice with phenol/chloroform (1:1), ethanol precipitated, and resuspended in TE buffer (0.5 μg/μl) for use as a riboprobe template. The transcription mixture, containing 1 μg of linearized plasmid template, 0.5 mM each of unlabeled nucleoside triphosphates, and 70 μCi of 10 mCi/ml ^{32}P-UTP in the presence of 1 unit per microliter of ribonuclease inhibitor, was incubated with 20 units of SP6 RNA polymerase for 90 min at 38 C. The mixture was then incubated at 37 C for 15 min with RNase-free deoxyribonuclease I to remove the DNA template. Phenol/chloroform-extracted RNA was ethanol precipitated and resuspended in TE buffer, pH 7.5, containing 1% 2-mercaptoethanol.

Hybridization, without a prehybridization step, was carried out overnight at 45 C in heat-sealable plastic bags containing 0.05–0.1 ml of the hybridization solution per square centimeter of membrane, using 4×10^5 cpm of RNA probe per milliliter of hybridization solution (40% formamide; 0.18 M NaCl; 10 mM sodium cacodylate, pH 7.0; 1 mM EDTA; 0.1% SDS; 10% dextran sulfate; and 300 μg/ml of calf-thymus DNA). After hybridization, membranes were washed as previously described (22), and hybridization was visualized by autoradiography. DNA probes were obtained by labeling pSP64:pX61 and pBR322:pPVX19 plasmids with ^{32}P-dCTP using a nick translation kit (BRL). Membranes were prehybridized for 2 h at 45 C in a solution of 50% formamide, 5× SSC, 5× Denhardt's reagent (15), 50 mM Na-phosphate (pH 6.5), 2.5 mM EDTA, 0.6% SDS, 5% dextran sulfate, and 100 μg/ml of denatured herring sperm DNA. Labeled probe (10^6 cpm/ml) was added directly to the prehybridization solution for hybridization and incubated overnight at 45 C. Membranes were washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature; once in 0.2× SSC, 0.2% SDS for 30 min at 37 C; once in 0.1× SSC, 0.2% SDS for 30 min at 55 C; then briefly rinsed in 0.1× SSC and air-dried prior to autoradiography.

NR-NASH. Plasmids pSP64:pX61 and pBR322:pPVX19 were biotinylated by nick translation in the presence of 50 μM bio-14-dATP (BRL) using a nick translation kit (BRL) with a DNase concentration of 4 pg/μl; incubation was carried out at 15 C for 90 min. Under these conditions, the biotinylated nucleotides substituted about 25% of the A residues. Unincorporated nucleotides were then removed by two ethanol precipitations in the presence of 2.5 M ammonium acetate. Biotinylated DNA was resuspended in 1× SSC and used at a concentration of 0.1–0.3 μg/ml.

Incubations were performed in heat-sealable plastic bags containing 0.05–0.1 ml of the hybridization solution per square centimeter of membrane. Membranes were prehybridized a minimum of 2 h at 42 C in a solution of 50% formamide, 5× SSC, 5× Denhardt's reagent, 25 mM Na-phosphate (pH 6.5), and 500 μg/ml of denatured herring sperm DNA. Hybridization was carried out overnight at 42 C in a solution of 45% formamide, 5× SSC, 1× Denhardt's reagent, 20 mM Na-phosphate (pH 6.5), 5% Dextran sulfate, 200 μg/ml of denatured herring sperm DNA, and 0.1–0.3 μg/ml of biotinylated cDNA probe.

After hybridization, membranes were washed as previously described (22); they were incubated for 20 min in 0.1 M Tris-HCl (pH 7.5) and 0.15 M NaCl containing streptavidin-alkaline phosphatase conjugate (BRL) diluted 1:2,000, then they were washed in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 0.05 M $MgCl_2$. Results were visualized by incubation in the dark in the same buffer containing 0.2 mg/ml of nitro-blue tetrazolium chloride and 0.1 mg/ml of 5-bromo-4-chloro-3-indolylphosphate.

RESULTS

Comparison between the nucleotide sequence of pX61 and the corresponding region of PVXcp, reported by Orman et al (18), showed 100% homology. The probe pX61 corresponds to the region between nucleotides 3,008 and 4,107 (Fig. 1). The pX61 sequence shows 78.5 and 78.8% homology to the corresponding sequences of PVX X3 (12) and PVX-S (24), respectively (Fig. 2). Probe pPVX19 corresponds to the region between nucleotides 2909 and 3845 of PVX RNA (Fig. 1). In that region, the nucleotide sequence showed 95.9 and 97.3% homology to the corresponding PVX sequence reported for PVX X3 and PVX-S, respectively, and 78.4% homology to PVXcp (Fig.2). The percentage of nucleotide changes in the nonoverlapping regions of probes 2,909–3,007 and 3,846–4,107 were 16 and 20% for the PVXcp and the European isolates, respectively. These results indicate that both probes, pX61 and pPVX19, derive from the central region of the PVX RNA genome and that they overlap in 838 nucleotides. The overlaps represent 76% of the pX61 toward the 3′ end and 89.5% of the pPVX19 towards the 5′ end.

Six experiments were conducted using leaf sap and the cp or

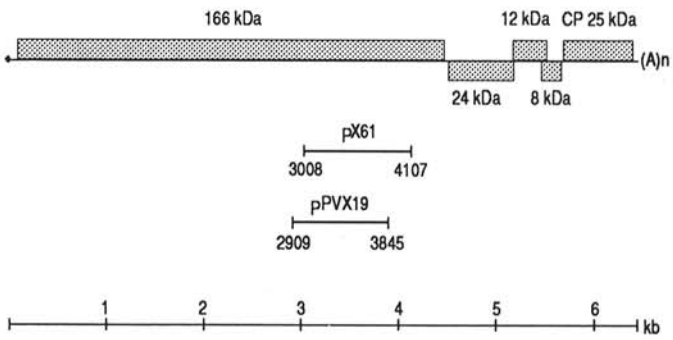

Fig. 1. Genomic organization of the potato virus X RNA and coordinates of the cDNA probes pX61 and pPVX19. Shaded boxes are open reading frames as reported by Orman et al (18). Arrowhead at 5′ end is m^7 GpppG cap structure. Poly (A) tail (A)n is at the 3′ end. CP = coat protein.

HB PVX^A isolates as positive controls. The PVX^O isolates included in the tests varied according to the experiment, but most were tested two to four times. All isolates were positive in the ELISA and indicated sufficient virus concentration in the leaf sap for the R-NASH and NR-NASH tests.

In R-NASH, the PVX^A isolates reacted more strongly than the PVX^O isolates with RNA transcribed from pX61. The cp isolate (homologous isolate) reacted more strongly than the HB (Fig. 3). The difference in detectability (detection limit) between them was not higher than two threefold dilution steps (TFDS). However, the difference between the HB isolate and the PVX^O isolates was at least 1 but usually 2–6 TFDS (Table 2). No difference was observed among serogroups I/II and IV from the PVX^O serotype. In addition to the differences observed in the detectability between isolates from PVX^A and PVX^O serotypes, another, more striking, difference was observed in the strength of the hybridization signal at lower dilutions, which was stronger for PVX^A isolates than for PVX^O isolates (Fig. 3).

Differences in the detectability of isolates from PVX^A and PVX^O serotypes were similar in NR-NASH using the DNA probe pX61 and in R-NASH using RNA pX61. However, the detectability of the homologous cp isolate and the PVX^O isolates in NR-NASH were approximately 1–3 and 1–9 TFDS lower, respectively, than in R-NASH (Fig. 3, Table 2). In reciprocal NR-NASH tests with DNA probe pPVX19, most PVX^O isolates (serogroups I/II and IV) reacted much more strongly than PVX^A isolates (Fig. 3, Table 2). Several PVX^O isolates, mostly from Bolivia, reacted as weakly as the PVX^A isolates (Table 2). These results were reconfirmed in R-NASH tests with DNA probe pPVX19 (Table 3). Differences observed in the detectability of isolates from PVX^A and PVX^O serotypes were reconfirmed when equal concentrations of purified PVX RNA from selected isolates were tested in R-NASH with the DNA probes pX61 and pPVX19 (Fig. 4).

The RNA probe pX61 did not react in R-NASH with: 1) four representative isolates from the three groups of strains of PVY (PVY^O, PVY^N, and PVY^C); 2) three isolates of Peru tomato virus-potato strain (potato virus V); 3) two isolates of potato virus A; 4) one isolate each of PVS, potato leafroll virus, potato virus M, Andean potato mottle virus, alfalfa mosaic virus, SB-22, tobacco ringspot virus, cucumber mosaic virus, and tobacco etch virus; and 5) three isolates of Andean potato latent virus (data not shown).

DISCUSSION

Results from DAS-ELISA indicated that there was sufficient virus present in leaf sap to detect differences among PVX^A and PVX^O isolates using each of the NASH variations. Because experiments were conducted at different times, some variability in virus concentration, and therefore in the reaction of a particular isolate, among experiments was expected.

The greater sensitivity of DAS-ELISA for detecting hetero-

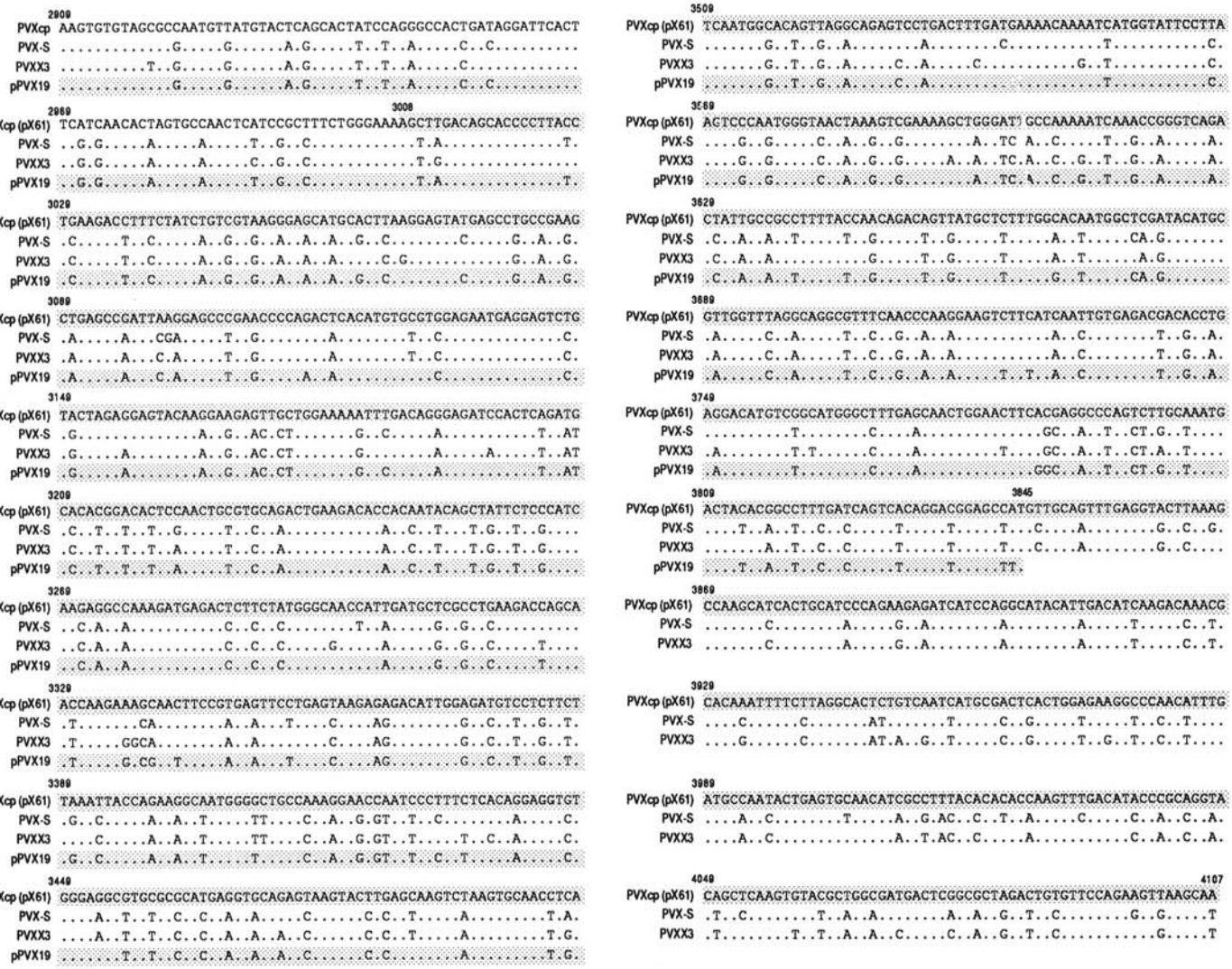

Fig. 2. Nucleotide sequence of the potato virus X (PVX) RNA genome covered by probes pX61 and pPVX19 (nucleotides 2909–4107). Upper line: nucleotide sequence of PVXcp according to Orman et al (18) and corresponding nucleotide sequence (3008–4107) for pX61 (dashed). Lower and bottom lines show only the differences in the sequences reported for PVX-S and PVX X3, by Skryabin et al (24) and Huisman et al (12), respectively, and in the corresponding nucleotide sequence (2909–3845) determined for pPVX19 (dashed).

logous isolates may be due to an appropriate combination of the coating IgG (cp) and the detecting IgG (HB) enzyme conjugate. The combination of a cp coating and a homologous cp conjugate does not properly detect a broad spectrum of PVX^O isolates by direct DAS-ELISA (8). Thus, the combination selected ensured high sensitivity to detect the broad spectrum of isolates used in this work.

When probe pX61 prepared from the PVXcp strain was used, it was shown that cp and HB isolates from the Andean serotype (PVX^A) differ from other isolates grouped in the common serotype PVX^O from the Andean region and from elsewhere. This evidence supports previous results obtained using monoclonal antibodies (8,14,25) and DNA probes prepared from a British isolate (3). In this work, for R-NASH with the RNA probe pX61, the difference in detectability between cp and HB isolates (PVX^A), and isolates from PVX^O serotypes, was usually 2–6 TFDS, suggesting that low virus concentrations might not be detected in plants infected with Andean isolates from the PVX^O serotype. For routine detection of PVX, the chances of escaping detection would be greater using NR-NASH. Results in this work and elsewhere (11) show that this technique does not yet seem to be as sensitive as R-NASH. This lower sensitivity affected the reproducibility of results with NR-NASH in this work when, in some experiments, some isolate concentrations were not as high as in other experiments. Although improvements in labeling and signal detection for NR-NASH, such as the chemiluminescence system (1,13), are under way, the utility of nonradioactive labeled probes for detecting PVX in leaf extracts remains to be determined.

Results of simultaneous tests with DNA probe pPVX19 were opposite to those obtained with DNA or RNA probe pX61 using the same samples. Most PVX^O isolates (from serogroups I/II and IV) reacted much more strongly in R-NASH or NR-NASH than the PVX^A isolates. The lower sensitivity of NR-NASH further lessens efficient detection. Several PVX^O isolates (serogroups I/II and IV), mostly from Bolivia, reacted as weakly as the PVX^A isolates. Probe pPVX19 cannot detect all the PVX^O isolates from the Andean region tested so far, indicating that there are divergent sequences in isolates within the PVX^O serotype. Probe pX61 detected these PVX^O isolates better than pPVX19, despite their differences in the coat protein gene from the PVX^A isolates. Therefore, the PVX^O isolates which have a closer geographic origin, have other areas in the nucleotide sequence that are more homologous with the PVX^A isolates than other PVX^O isolates

TABLE 3. Reaction of selected PVX isolates in radioactive nucleic acid spot hybridization tests using DNA probe pPVX19 and RNA probe pX61 prepared from a PVX^O and a PVX^A isolate, respectively.

Isolate	Serotype (serogroup)	pPVX19	pX61
GUA2	X^O (IV)	9[a]	4
Luc 10	X^O (IV)	9	5
8	X^O (IV)	8	4
3	X^O (IV)	8	5
Luc 5-2	X^O (IV)	8	6
Luc 3-1	X^O (IV)	8	7
Luc 6-1	X^O (IV)	8	7
59	X^O (IV)	6	4
2	X^O (I/II)	7	5
3Bo	X^O (IV)	6	6
2Bo	X^O (IV)	3	6
29Bo	X^O (II)	3	7
27Bo	X^O (I/II)	1	6
46Bo	X^O (I/II)	1	5
73Bo	X^O (I/II)	1	8
20	X^O (II)	1	7
HB	X^A(III)	1	>9
cp	X^A(III)	1	>9
Healthy		0	0

[a] Numbers assigned to reciprocal (× 1,000) of highest dilution of sap extracted from *Nicotiana glutinosa* leaves healthy or infected with PVX isolates, at which a reaction was noted after visual observation: 1 = 0.08, 2 = 0.24, 3 = 0.73, 4 = 2.19, 5 = 6.56, 6 = 19.7, 7 = 59.1, 8 = 177, 9 = 531.

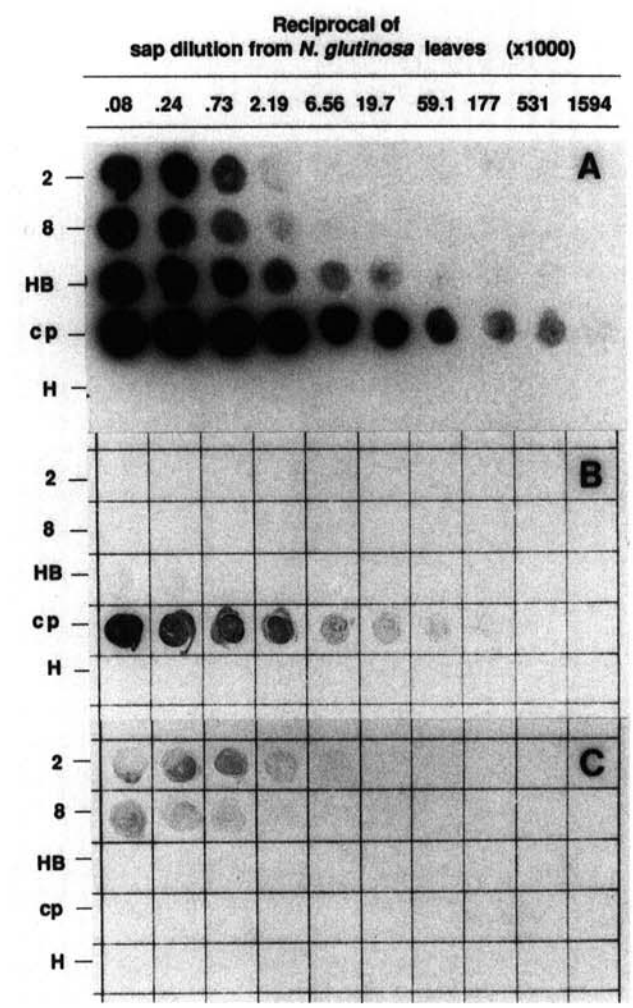

Fig. 3. Differential detection of potato virus X (PVX) serotypes by **A**, radioactive nucleic acid spot hybridization using RNA probe pX61, **B**, nonradioactive nucleic acid spot hybridization using DNA probe pX61, or **C**, pPVX19. Isolates 2 and 8 are PVX^O serotype (serogroups I/II and IV, respectively), and isolates HB and cp are PVX^A serotype (serogroup III). H is healthy sap.

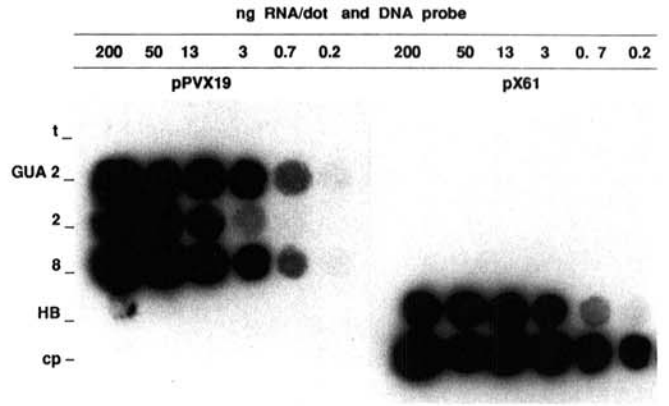

Fig. 4. Differential detection of potato virus X (PVX) serotypes PVX^O (isolates GUA 2, 2, and 8) and PVX^A (isolates HB and cp) by radioactive nucleic acid spot hybridization tests using DNA probes pPVX19 and pX61. Samples are dilutions of purified RNA of PVX isolates. t = purified transfer RNA as control.

not geographically related.

Although no European isolates from the PVX^O serotype were tested with the RNA probe pX61, it seems to detect isolates from PVX^O serotype better than the DNA probe pPVX19 detects isolates in the PVX^A serotype (3). Therefore, the RNA probe pX61 seems to detect a broader spectrum of PVX isolates.

Orman et al (18) demonstrated that the PVXcp complete genomic sequence differs significantly from the ones reported for two European isolates, the X3 strain (12) and the Russian strain PVX-S (24). Although the two European strains showed 97.1% nucleotide homology between them, they showed only about 77% overall homology with PVXcp. From recent studies, it is believed that these two European isolates belong to the PVX^O serotype (8). The two probes used detected differences between PVX^O and PVX^A serotypes in the nucleotide sequence of the central part of the genomic RNA of PVX, towards the 3′ end of the open reading frame (ORF) 1 that encodes for 166-kDa protein apparently involved in viral replication (12). The differences detected are not in areas of significant divergence between the nucleotide sequence of PVXcp and the two European isolates. Those differences (18) are clustered between nucleotides 1490 and 1928 in ORF 1; between 5650 and 5920 in ORF 5, which encodes the coat protein; and in the entire ORF 4, which encodes an 8-kDa polypeptide that might fulfill a function in viral spread (12). Probe pX61 and probe pPVX19, especially, detect differences in nucleotide sequences between PVX^O and PVX^A in a region of low divergence. The two probes hybridize within a region that is relatively conserved but does show 22% nucleotide changes. pX61 hybridizes in the putative region of the polymerase sequence (3968–4073), which is expected to be a conserved region. In comparison, pX61 covers a larger region (262 nucleotides) of lower divergence than pPVX19 (99 nucleotides), probably allowing hybridization with isolates of the heterologous serotype. This explains the ability of the pX61 to detect a broader spectrum of PVX isolates than the pPVX19. It is expected that the divergence among some PVX^O isolates from the Andean region and PVX^A is still lower than among PVX^O isolates from elsewhere and PVX^A.

In our R-NASH tests, the hybridization signal of the heterologous serotype with the DNA probes using purified RNA was weaker than with the RNA probes using leaf sap. This enhanced the differences between the PVX^A and PVX^O isolates. Several factors could be involved: higher concentration of viral RNA, coat protein binding of PVX RNA to the filter due to using leaf sap, degradation of PVX RNA during the purification process, or better reactivity of the RNA probe for the heterologous serotype.

Hopp et al (11) prepared a 500-bp DNA probe (pX1) from the PVXcp isolate. Apparently, this probe was not tested against a broad spectrum of PVX isolates, but it could be more specific than pX61 because of its smaller size. They also found R-NASH to be more sensitive than the NR-NASH but reported similar sensitivities for NR-NASH and ELISA. In our work, the sensitivities of DAS-ELISA, R-NASH, and NR-NASH were similar when using the homologous PVXcp isolate, but the sensitivity of ELISA was higher with heterologous isolates from the Andean region.

The differences among PVX^A and PVX^O isolates confirmed in this report stress the importance of using the appropriate antibodies or probes in detection techniques, especially for breeding programs for resistance and quarantine purposes. Probe pX61 will be very valuable for the quarantine detection of the PVX HB pathotype (7) from the PVX^A serotype that breaks the immunity to PVX in potato (17). This pathotype is found only in Bolivia at the present time (8).

LITERATURE CITED

1. Audy, P., Parent, J.-G., and Asselin, A. 1991. A note on four nonradioactive labeling systems for dot hybridization detection of potato viruses. Phytoprotection 72:81-86.
2. Baulcombe, D. C., Boulton, R. E., Flavell, R. B., and Jellis, G. J. 1984. Recombinant DNA probes for detection of viruses in plants. Pages 207-213 in: Proc. Br. Crop Prot. Conf.
3. Baulcombe, D. C., and Fernandez-Northcote, E. N. 1988. Detection of strains of potato virus X and of a broad spectrum of potato virus Y isolates by nucleic acid spot hybridization (NASH). Plant Dis. 72:307-309.
4. Baulcome, D. C., Flavell, R. B., Boulton, R. E., and Jellis, G. J. 1984. The sensitivity and specificity of a rapid nucleic acid spot hybridization method for the detection of potato virus X in crude sap samples. Plant Pathol. 33:361-370.
5. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
6. Eweida, M., Sit, T. L., and AbouHaidar, M. G. 1989. Molecular cloning of the genome of the carlavirus potato virus S: Biotinylated RNA transcripts for virus detection in crude potato extracts. Ann. Appl. Biol. 115:253-261.
7. Fernandez-Northcote, E. N. 1990. Variability of PVX and PVY and its relationship to genetic resistance. Pages 131-139 in: Control of Virus and Virus-like Diseases of Potato and Sweet Potato. Report of the 3rd Planning Conference 1989. International Potato Center, Lima, Peru.
8. Fernandez-Northcote, E. N., and Lizarraga, C. 1991. Distribución geográfica de serotipos del virus X de la papa. Fitopatologia 26:13-18.
9. Hammond, J., and Lawson, R. H. 1988. An improved purification procedure for preparing potyviruses and cytoplasmic inclusions from the same tissue. J. Virol. Methods 20:203-217.
10. Hellmann, G. M., Shahabuddin, M., Shaw, J. G., and Rhoads, R. E. 1983. Molecular cloning of DNA complementary to tobacco vein mottling virus RNA. Virology 128:210-220.
11. Hopp, H. E., Giavedoni, L., Mandel, M. A., Arese, A., Orman, B., Bravo Almonacid, F., Torres, H. N., and Mentaberry, A. N. 1988. Biotinylated nucleic acid hybridization probes for potato virus detection. Arch. Virol. 103:231-241.
12. Huisman, M. J., Linthorst, H. J. M., Bol, J. F., and Cornelissen, B. J. C. 1988. The complete nucleotide sequence of potato virus X and its homologies at the amino acid level with various plus-stranded RNA viruses. J. Gen. Virol. 69:1789-1798.
13. Kanematsu, S., Hibi, T., Hashimoto, J., and Tsuchizaki, T. 1991. Comparison of nonradioactive cDNA probes for detection of potato spindle tuber viroid by dot-blot hybridization assay. J. Virol. Methods 35:189-197.
14. Lizarraga, C., and Fernandez-Northcote, E. N. 1989. Detection of potato viruses X and Y in sap extracts by a modified indirect enzyme-linked immunosorbent assay on nitrocellulose membranes (NCM-ELISA). Plant Dis. 73:11-14.
15. Maniatis, T. A., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
16. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. 12:7035-7056.
17. Moreira, A., Jones, R. A. C., and Fribourg, C. E. 1980. Properties of a resistance-breaking strain of potato virus X. Ann. Appl. Biol. 95:93-103.
18. Orman, B. E., Celnik, R. M., Mandel, A. M., Torres, H. N., and Mentaberry, A. N. 1990. Complete cDNA sequence of a South American isolate of potato virus X. Virus Res. 16:293-306.
19. Owens, R. A., and Diener, T. O. 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-672.
20. Salazar, L. F., Balbo, I., and Owens, R. A. 1988. Comparison of four radioactive probes for the diagnosis of potato spindle tuber viroid by nucleic acid spot hybridization. Potato Res. 31:431-442.
21. Salazar, L. F., Owens, R. A., Smith, D. R., and Diener, T. O. 1983. Detection of potato spindle tuber viroid by nucleic acid spot hybridization: Evaluation with tuber sprouts and true potato seed. Am. Potato J. 60:587-597.
22. Salazar, L. F., and Querci, M. 1992. Detection of viroids and viruses by nucleic acid probes. Pages 129-144 in: Techniques for the Rapid Detection of Plant Pathogens. J. Duncan and L. Torrance, eds. Blackwell Scientific Publications.
23. Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463-5467.
24. Skryabin, K. G., Kraev, A. S., Morozov, S. Y., Rozanov, M. N., Chernov, B. K., Lukasheva, L. I., and Atabekov, J. G. 1988. The nucleotide sequence of potato virus X RNA. Nucleic Acids Res. 16:10929-10930.
25. Torrance, L., Larkins, A. P., and Butcher, G. W. 1986. Characterization of monoclonal antibodies against potato virus X and comparison of serotypes with resistance groups. J. Gen. Virol. 67:57-67.