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Article abstract

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A note on four nonradioactive labeling systems for dot hybridization detection of potato viruses

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Complementary DNA clones of genomic RNAs of potato (*Solanum tuberosum*) viruses S (PVS), X (PVX) and Y (PVY) were produced and tested for their capacity to hybridize with various plant virus RNAs. PVS clone S12 and PVX clone X6 were found to be very specific to PVS and PVX RNA respectively, whereas PVY clone Y10 strongly hybridized with PVY RNA and weakly with PVS RNA. Four commercial, nonradioactive systems of nucleic acid labeling and detection were compared to the usual ^{32}P-labeled probe using dot hybridization experiments. Colorimetric detection of digoxigenin-labeled DNA probes gave a level of sensitivity of 1 ng of virions (60 pg of RNA), similar to autoradiography of ^{32}P-labeled probes. Sulfonated, biotinylated and peroxidase-labeled probes were slightly less sensitive, allowing detection of 600 pg of viral RNA.

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Des clones d'ADN complémentaire ont été fabriqués à partir des ARN génomiques des virus S (PVS), X (PVX) et Y (PVY) de la pomme de terre (*Solanum tuberosum*). Les clones ont été sélectionnés pour leur spécificité par l'hybridation avec divers ARN viraux. Les clones S12 de PVS et X6 de PVX se sont avérés très spécifiques à l'ARN de PVS et PVX respectivement, alors que le clone Y10 de PVY a hybridé fortement à l'ARN du PVY et faiblement à l'ARN du PVS. Quatre systèmes commerciaux non radioactifs de marquage des acides nucléiques et de détection ont été comparés entre eux et avec le marquage radioactif traditionnel de la sonde au ^{32}P. La détection colorimétrique de sondes d'ADN marquées à la digoxygénine permet de déceler 1 ng de virions (60 pg d'ARN), soit une sensibilité du même ordre que l'autoradiographie avec des sondes marquées au phosphore radioactif. Les sondes sulfonées, biotinylées et marquées à la peroxydase ont été moins sensibles en permettant la détection de 600 pg d'ARN viral.

Potato viruses S (PVS), X (PVX) and Y (PVY) cause economically important losses in potato (*Solanum tuberosum* L.) production. Reductions in tuber yields of infected plants of 30%, 37% and 80% have been reported (de Bokx and Huttinga 1981;Wright 1970, 1977). PVY and some PVS isolates that are aphid transmitted (Kostiw 1979; Wardrop *et al.* 1989), and PVX that is easily mechanically transmitted, often cause rapid reinfection of virus-free potato seed plants in the field (McDonald 1987). Detection of early viral infections in seed stocks is important to maintain high standards required by certification agencies.

Serological techniques, specially ELISA, are commonly used to diagnose viral diseases in potato (Zink 1991). Recent advances in molecular biology have led to the development of nucleic acid probes for the sensitive detection and identification of plant viruses. These probes are at least as sensitive as ELISA (Barbara *et al.* 1987; Roy *et al.* 1988; Stenger *et al.* 1987; van der Vlugt *et al.* 1988) and, in some cases, a ten-fold increase in comparative sensitivity has been reported (Bijaisoradat and

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Kuhn 1988; Eweida *et al.* 1990; Pesic and Hiruki 1988; Varveri *et al.* 1987). Molecular hybridization methods have been used to detect various potato viruses (Baulcombe and Fernandez-Northcote 1988; Baulcombe *et al.* 1984; Monis and de Zoeten 1990b; Waterhouse *et al.* 1986). Unfortunately, molecular probes are not widely used because they are usually labeled with ^{32}P-nucleotides. Radiation safety, rapid decay of some radioisotopes and storage of radioactive waste are major restrictions for routine diagnostic procedures (Hopp *et al.* 1988). Nonradioactive nucleic acid labeling and detection approaches have recently emerged as alternatives to the use of radioactivity in nucleic acid hybridization experiments (Eweida *et al.* 1989; Hopp *et al.* 1988; Roy *et al.* 1988). The objectives of the present study were to prepare cDNA clones from PVS, PVX and PVY RNAs and to use them as probes to evaluate new commercial, nonradioactive systems of labeling.

Potato plants infected with local isolates of PVS, PVX and PVY were obtained from Agriculture Canada (La Pocatière, Québec). Tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV), strain U_1, were purified from infected *Nicotiana tabacum* L. cv. Turkish Samsun according to Gooding and Hebert (1967). Barley stripe mosaic virus (BSMV) was purified from infected *Hordeum vulgare* L. cv. Sophie according to Jackson and Brakke (1973). PVX, PVY and PVS were maintained under greenhouse conditions in *N. tabacum* cv. Xanthi-nc (PVX and PVY) and potato cv. Superior (PVS) plants. Viruses were purified from infected leaves as described by Shepard (1972) except that urea (1 M) was added to the buffer after each polyethylene glycol precipitation for PVS and PVY. Viral RNAs were isolated from purified virions by heating at 50°C for 5 min in the presence of 0.5% sodium dodecyl sulfate (SDS). RNA was extracted twice with an equal volume of phenol:chloroform (1:1) and precipitated with ethanol.

The genomes of PVS, PVX and PVY consist of a single-stranded positive sense RNA of 7.5, 6.4 and 9.7 kb, respectively, and contain a 3'-terminal polyadenylated region (Huisman *et al.* 1988; Monis and de Zoeten 1990a; Turpen 1989). First strand synthesis of PVS, PVX and PVY cDNAs was carried out with Moloney murine leukemia virus reverse transcriptase (Pharmacia) using oligoprimers (6 bp) for PVX RNA and oligo-dT12-18 primer for PVS and PVY RNAs. Second strand synthesis was performed according to Gubler and Hoffman (1983) using a commercial kit (cDNA synthesis kit, Pharmacia). Virus cDNAs were blunt-ended with the Klenow fragment of DNA polymerase I, ligated to *Eco*RI linkers with T4 DNA ligase and inserted into the *Eco*RI site of λgt10 for PVX or in ß-galactosidase lac-Z gene of pT7T3 18U (Pharmacia) for PVS and PVY. Forty PVX-λgt10 plaques chosen at random from a phage library and multiplied by plating on *Escherichia coli* C600. Their cDNA inserts were digested with *Eco*RI and subcloned into pUC 13. Competent *E. coli* NM522 or JM103 cells (Mandel and Higa 1970) were transformed by the plasmids containing cDNAs. With each viral cDNA made by oligo (dT) priming, we isolated approximately 40 ampicillin-resistant and ß-galactosidase negative plasmids using isopropyl-ß-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal). The presence and size of inserts was determined by plasmid isolation (Birnboim and Doly 1979) followed by *Eco*RI digestion and electrophoresis on agarose gel with DNA size markers (123 bp DNA ladder, Bethesda Research Laboratories). Clones with an insert size of approximatively 3.0 kb for PVS and PVY and 1.0 kb for PVX were selected and ^{32}P-labeled to test their specificity against a few purified plant viruses using nucleic acid dot hybridization (Fig. 1).

To compare detection systems, plasmids containing viral cDNAs were radioactively labeled by random priming (Feinberg and Vogelstein 1983) with α^{32}P-dCTP (oligolabeling kit, Pharmacia). Digoxigenin-11-dUTP (Boehringer Mannheim) and biotin-14-dATP (Bethesda Research Laboratories) were also incorporated into plasmids respectively by random priming and nick translation, whereas sulfonation (FMC BioProducts) and direct peroxidase labeling of DNA (Amersham) were performed according to the instructions of the manufacturers.

Purified virus solutions were serially diluted with 1% SDS, boiled 5 min and spotted (5 µL) onto nylon membranes (Nytran, Schleicher and Schuell). Viral RNAs were exposed to UV (transilluminator TS-15 Chromato-vue) for 3 min at 20 cm from the source (Church and Gilbert 1984). Hybridization conditions and

detection were performed according to the manufacturers' directions for nonradioactive systems. With ^{32}P-labeled probes, prehybridization was carried out for 4 h in sealed polyethylene bags at 42°C in 50% formamide (Sigma), 2X Denhardt's solution (1X is 0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin, all from Sigma), 5X SSC (1X is 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 10% dextran sulfate (Sigma), 0.1% SDS and 0.1 mg/mL of denaturated salmon-sperm DNA (Sigma). Hybridizations with ^{32}P-labelled DNA were performed at 42°C for 16 h in prehybridization mixture, except that salmon-sperm DNA was at 0.5 mg/mL. These hybridizations were performed in the presence of 20 ng of labeled probes per cm^2 of membrane. ^{32}P-labeled probes had a specific activity of at least 1.5 x 10^6 cpm/µg DNA. Membranes were washed twice, 10 min each, in 2X SSC and 0.1% SDS at room temperature, 30 min in 2X SSC and 0.1% SDS at 42°C and twice (30 min each) in 0.1X SSC and 0.1% SDS at 42°C. Autoradiography was for 6 to 8 h at -70°C with an intensifying screen using Kodak XAR-2 films. Each of the experiments was repeated at least twice.

Radioactively labeled PVS clone S12, PVX clone X6 and PVY clone Y10 were tested for their specificity against a few plant viruses (Fig. 1). PVS clone S12 (Fig. 1B) and PVX clone X6 (Fig. 1C) were quite specific, hybridizing strongly with PVS or PVX RNA but poorly with the heterologous viral RNAs. PVY clone Y10 hybridized strongly with PVY RNA but also hybridized to BSMV, ToMV, TMV, PVX and PVS (Fig. 1A). Higher specificity was obtained using more stringent conditions but PVS RNA still cross-hybridized with PVY probe Y10, but TMV and BSMV were not tested (Fig. 2A). To potentially decrease the unwanted hybridization, the inserted fragment of 1.6 kb obtained by *Eco*RI digestion of plasmid Y10 was purified and ^{32}P-labeled. The same hybridization specificity was observed. In addition, this fragment bound to oligo(dT)-cellulose revealing the presence of a poly(A) track or a very A-rich region in PVY plasmid Y10. However, this does not explain the hybridization with polyadenylated PVS RNA since no cross-hybridization was observed with polyadenylated PVX RNA. Pairwise computer alignments between available nucleotide sequences of PVY (Robaglia *et al.* 1989) and PVS (McKenzie *et al.* 1989) revealed a 72% homology for a region of 93 residues located at 2500 (PVY) and 2800 (PVS) residues downstream their 3' end (data not shown). Homology even reached 81% for a stretch of 26 nucleotides. This could be sufficient to give significant hybridization signal between PVS RNA and PVY Y10 probe. According to the size of the PVS fragment cloned, this region is absent from the PVS S12 probe. That is probably why PVS S12 probes did not hybridize with PVY RNA. For diagnostic purposes, PVY Y10 probe should be modified to eliminate this region of strong homology with PVS RNA.

The sensitivity of four commercial systems of probe labeling and detection was compared to the usual ^{32}P-labeled DNA method using dot hybridization procedures. Two of these systems were based on a colorimetric reaction from alkaline phosphatase-linked antibodies that were bound with either antigenic digoxigenin molecules (Boehringer Mannheim) or

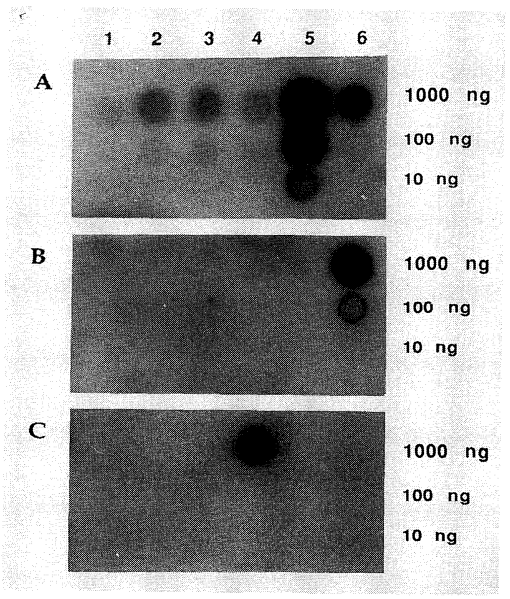

Figure 1. Dot blot hybridization of viral RNAs using ^{32}P-labeled (**A**) PVY probe Y10, (**B**) PVS probe S12 and (**C**) PVX probe X6. Purified BSMV (1), ToMV (2), TMV (3), PVX (4), PVY (5) and PVS (6) were serially diluted, denatured in the presence of SDS and spotted onto nylon sheets. Hybridizations were performed in the presence of 20 ng of labeled probes per cm^2 of membrane. ^{32}P-labeled probes had a specific activity of at least 1.5 x 10^6 cpm/µg DNA. Autoradiography was for 8 h with XAR-2 Kodak films at -70°C.

sulfone groups (FMC BioProducts) attached to modified nucleotides. The two others involved detection with X-ray films of luminescence emitted from enzymatic reactions using streptavidin-alkaline phosphatase conjugates on a biotin-labeled probe (Bethesda Research Laboratories) or a peroxidase-labeled probe (Amersham). Hybridizations were performed in the presence of 8 ng of labeled probes per cm^2 of membrane. The ^{32}P-labeled probe had a specific activity of 4.5 x 10^8 cpm/µg DNA.

The detection limit using ^{32}P-labeled probe Y10 was close to 1.0 ng of PVY virions (very faint spot) or 60 pg of viral RNA (assuming 6% RNA per particle) (de Bokx and Huttinga 1981)

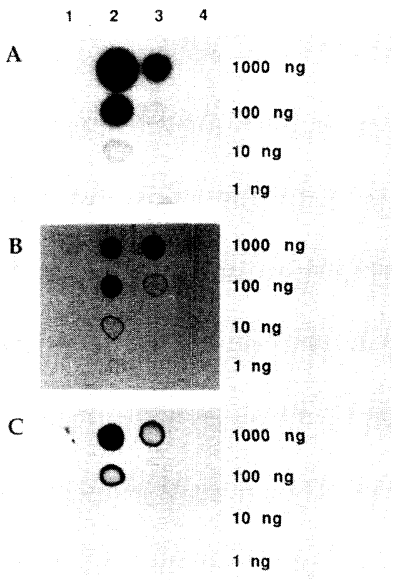

Figure 2. Dot blot hybridization of various viral RNAs using (**A**) ^{32}P-, (**B**) digoxigenin, and (**C**) peroxidase- labeled PVY cDNA probe Y10. Purified ToMV (1), PVY (2), PVS (3) and PVX (4) were serially diluted, denatured in the presence of SDS and spotted onto nylon sheets. Hybridization and detection of nonradioactive probes were performed according to the manufacturers' instructions. Standard procedures were used for radioactive probes. Hybridizations were performed in the presence of 8 ng of labeled probes per cm^2 of membrane. The ^{32}P-labeled probe had a specific activity of 4.5 x 10^8 cpm/µg DNA. Autoradiography was for 6 h with XAR-2 Kodak films at -70°C. Hybridized peroxidase-labeled probes were detected by enhanced chemiluminescence using a 60 min exposure to a XAR-2 Kodak film at 22°C. Hybridization signals of digoxigenin-labeled probes were developed during 6 h at 22°C.

for a 6-h exposure (Fig. 2A). The same level of detection was obtained using colorimetric-digoxigenin labeling after 6 h of enzymatic development (Fig. 2B). Similar results were obtained with the PVS probe S12 and PVX probe X6 (data not shown). In the latter case, however, the digoxigenin probe concentration was raised to 25-50 ng/mL to obtain a sensitivity equivalent to radioactive labeling.

Luminescent-peroxidase Y10 probe was 5 to 10 times less sensitive than colorimetric detection of digoxigenin-labeled probe (Fig. 2C). Similar results were obtained with biotin-labeled probes (data not shown). In spite of their lower level of sensitivity, the two systems based on photographic detection of luminescent DNA probes, have the advantage of being completely independent from the color of tissue samples. Such colored samples can create significant problems with colorimetric methods using dot hybridization of plant extracts (Eweida *et al.* 1989; Hopp *et al.* 1988;).

At typical probe concentration, sulfonated probes were slightly less sensitive (data not shown) and required more time-consuming manipulations. In addition, sulfonated X6 probes could be as sensitive as digoxigenin-labeled probes if they were used at concentration of 600-1000 ng/mL (24 times more concentrated than digoxigenin probes). Sulfonated probes have also been found less sensitive than biotinylated probes in dot blot hybridization procedures (Johansen *et al.* 1989). Because of the presence of biotin-like components in plant material (Cuppels *et al.* 1990; Hull and Al-Hakim 1988), sulfonated probes could possibly yield a better level of detection in plant extracts because of lower background reactions. Such problems arising with biotinylated probes could be alleviated when DNAs of plant extracts are purified (Roy *et al.* 1988).

Since the early 1980s, nonradioactive probes emerged slowly as an alternative to the use of radioactivity to label nucleic acids. The sensitivity of digoxigenin-labeled probes and the speed of detection have been reported to be comparable to those of ^{32}P-labeled probes in dot blot assays and appeared to be superior to other nonradioactive systems (Lion and Haas 1990). However, for plant virus detection, a biotin probe gave a sensitivity of detection similar to a ^{32}P-labeled probe (Roy

et al. 1988). Moreover, the detection level using biotinylated RNA probes has been reported to be high enough to detect femtograms of PVS RNA (Eweida *et al.* 1989).

In this study, we have tested four commercial, nonradioactive DNA labeling kits for dot hybridization detection of PVS, PVX and PVY RNAs. As little as 60 pg of viral RNAs could be detected within 6 h of enzymatic development using colorimetric-digoxigenin labeling system. This detection level was similar to that obtained with ^{32}P-labeled probes for the same exposure time. The other systems of labeling gave a lower level of sensitivity. However, the enhanced peroxidase chemiluminescence system (Amersham), involving direct labeling of DNA probe (Renz and Kurz 1984), was found to be the most convenient approach for large-scale detection of specific nucleic acid sequences. Probe labeling with peroxidase is very short (10 min) and detection of probes after washings can be obtained within 60 min. Only purified virus was used, and the utility of the nonradioactive labelled probes to detect virus in plants remains to be determined.

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